Analysis of the Role of Caspase 2 in Apoptosis

by

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Declaration

The thesis has been composed by myself and the experiments reported in this thesis were carried out by myself unless stated otherwise in the text.

Britta Dost

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ABSTRACT OF THESIS

(Regulation 3.5.13)

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of Thesis Analysis of the Role of Caspase 2 in Apoptosis

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ptosis is a process that deletes cells from living tissues. It constitutes a stereotyped sequence of events culminate in the fragmentation of the cell and the ingestion of its remains by mononuclear phagocytes. se structural changes are the result of proteolytic cleavage of key cellular substrates and reflect common inal effector events. Members of a family of cysteine proteases, called caspases, are activated during otosis and are implicated as key mediators of these proteolytic events. Caspase 2/human ICH-1/mouse OD-2 was identified as a member of the caspase family based on sequence similarity to prototype caspases 03 and caspase 1/ICE. Its specific role in apoptosis has not yet been clearly defined.

rder to define the role of caspase 2 in apoptosis, three rabbit polyclonal antibodies were raised against erially expressed recombinant caspase 2 polypeptides. Two anti-caspase 2 antibodies were successfully ed and the specificity of those antibodies to the prodomain and the small subunit was confirmed by unoblot and ELISA analysis. Both antibodies proved unsuitable for the use as immunocytochemical and unohistochemical reagents, were however valuable tools in Western blotting.

ommercially available anti-caspase 2 monoclonal antibody, generated against recombinant caspase 2, was vn by immunoblot analysis to detect a cellular protein of the appropriate molecular weight for procaspase his antibody was used to investigate caspase 2 expression by immunoblot analysis in various human es and cell lines. Protein expression was found at various levels in all 11 tissues extracts analysed and unoreactivity was localised within the cytosol in several human tissues. Activation of caspase 2 in otosis was analysed in two human cell lines in response to three different apoptotic stimuli: etoposide, osporine and Fas ligation. Immunoblotting with the Transduction Laboratories monoclonal anti-caspase tibody revealed no change in the quantity or molecular weight of the detected epitope during apoptosis. mation obtained from immunohistochemistry or Western blotting using the Transduction Laboratories caspase 2 monoclonal antibody was proved to be irrelevant to the study of caspase 2, as the protein cted by this antibody was shown not to be caspase 2.

tern blotting analysis with the two polyclonal anti-caspase 2 antibodies raised and two other non-mercial antibodies to caspase 2 established activation of caspase 2 during apoptosis in response to oside, anti-Fas and staurosporine treatment. The kinetics of caspase 2 activation were shown to be similar aspase 3 activation and PARP cleavage.

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Abbreviations

AIF	apoptosis-inducing factor
ApaF-1	apoptotic protease activation factor
AMPS	ammonium persulphate
ATP	adenosine triphosphate
BH	Bcl-2 homology domain
BSA	bovine serum albumin
CARD	caspase recruitment domain
CASH	caspase homolgue
caspase	cysteine protease with aspartic acid cleavage specificity
casper	caspase 8 related protein
CED	cell death abnormal
CHO	ketone
CMK	chloromethyl ketone
CRADD	caspase and RIP adater with death domain
CTL	cytotoxic T lymphocyte
DAB	3,3-diaminobenzidine
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DEVD	Asp-Glu-Val-Asp
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
EDTA	diamino tetra acetic acid
ELISA	enzyme-linked immunosorbant assay
FADD	Fas-associating protein with death protein
FLAME-1	FADD-like antiapoptotic molecule
FLICE	FADD-like interleukin-1β converting enzyme
FLIP	FLICE-inhibitory protein
HRP	horse raddish peroxidase
HPLC	high performance liquid chromatography
IAP	inhibitor-of-apoptosis
ICE	interleukin-1β converting enzyme
ICAD	inhibitor of caspase-activated desoxyribonuclease
ICH-1	ICE and CED3 homolog 1
I-FLICE	inhibitor of FLICE
Ig	immunoglobulin
IL-1β	interleukin-1β
IPTG	isopropylthio-β-D-galactopyronoside
kDa	kilodalton
lacZ	*E.coli* β galactosidase gene
LPS	lipopolysaccharide
MALDI-TOF	matrix-assisted laser desorption/ionisation time of flight mass spectrometry
mRNA	messenger RNA

MORT1	mediator of receptor induced toxicity 1
Nedd2	NPC expressed developmentally downregulated gene 2
OD	optical density
ODP	O-phenylenediamine dihydrochloride
PARP	poly(ADP) ribose polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
QACRG	Gln-Ala-Cys-Arg-Gly
RAIDD	RIP-associated ICH-1/CED3 homolgous protein with death domain
RIP	receptor interacting protein
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris-acetate/EDTA electrophoresis buffer
TBE	Tris-borate/EDTA electrophoresis buffer
TE	Tris/EDTA buffer
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TMD	tetramethylbenzidine free base
TNF	tumour necrosis factor
TNFR1	tumor necrosis factor receptor 1\
TRADD	TNFR-1 associated death domain protein
UV	ultraviolet
x-Gal	5-bromo-4-chloro-3-indyl-β-D-galactopyranoside
YVAD	Tyr-Val-Ala-Asp

1. INTRODUCTION

1.1 DEFINITION OF APOPTOSIS

The word 'apoptosis' is derived form a Greek word describing the process of leaves falling from trees or petals from flowers. It was introduced to describe a morphologically distinct form of cell death whereby single cells are deleted from living tissues (Kerr *et al*, 1972). Apoptosis is now known to be effected by a common set of endogenous events, amongst them the activation proteases, called caspases, because they contain a cysteine residue at their active site and selectively cleave peptide bonds adjacent to aspartic acid residues. One of these, caspase 2 (ICH-1), is the subject of this thesis.

1.2 MORPHOLOGY OF APOPTOSIS

Cells undergoing apoptosis exhibit a characteristic set of morphological changes, which occur in apoptosis regardless of the cell type (reviewed by Arends *et al*, 1994).

In the nucleus events of apoptosis begin with the condensation of chromatin, which progressively becomes more condensed and either forms a single chromatin aggregate or collapses against the nuclear membrane and forms several separate aggregates in hemilunar caps. Subsequently the nuclear membrane undergoes convolutions and the condensed nucleus fragments to form spheres of dense nuclear material. These nuclear changes are associated with a redistribution of nuclear pores, disassembly of the nuclear lamina and endonuclease dependent DNA fragmentation (Wyllie *et al*, 1980A; Lazebnik *et al*, 1993; Enari *et al*, 1998). The DNA is cleaved first into large (50-300kb) fragments, referred to as 'domain' cleavage, followed by cleavage in the internucleosomal linker regions, which are spaced regularly at approximately 180-200 base pair (bp) intervals along eukaryotic chromatin, into single and multiple nucleosomes fragments (Wyllie, 1980B). These chromatin fragments can be observed by agarose gel electrophoresis as a DNA ladder pattern of bands of intiger multiples of about 200bp.

Cytoplasmic features of apoptosis include a loss of cell volume, aggregation of organelles and disintegration of organelle structure. The endoplasmic reticulum dilates and form expanded vesicles, which sometimes fuse with the plasma membrane giving

it a pitted appearance. Other cytoplasmic organelles remain largely unaffected. The dying cell violently extrudes and withdraws finger-like extensions (blebs), shrinks in cell volume and eventually fragments into membrane bound apoptotic bodies of various sizes some, containing condensed chromatin.

1.3. SIGNIFICANCE OF APOPTOSIS

"No self-respecting cell would be seen dead other than by apoptosis these days" (Allen *et al*, 1995). Apoptosis occurs in most , if not all, multicellular animals, plants, probably even fungi, and possibly some unicellular organisms including *Trypanosoma cruzi* and *Tetrahymena thermophilia* (Cornillon *et al*, 1994; Davis *et al*, 1992; Ameisen *et al*, 1995). It thus seems that many cells, regardless of the phylogenetic origin or physiological specialisation may ultimately die by apoptosis.

Apoptosis occurs under physiological conditions during embryogenesis and morphogenesis in which it is involved in sculpturing of organs and deleting unneeded structures. It is essential for tissue atrophy and normal homeostasis of multicellular organisms by removing redundant cells, which include cells that have fulfilled their lifespan, are non-functional or abnormal. It is also a means to precisely regulate cell numbers and is particular important in the developing nervous system (Raff *et al*, 1993) and in the development and effective functioning of the immune system (reviewed by Williams, 1994). Apoptosis is also triggered in response to pathological stimuli including viral invasion and is thus an important mechanism for removal of infected and potentially hazardous cells. The crucial importance of apoptosis, however, becomes clear from well-studied examples of animal development (reviewed by Jacobson *et al*, 1997). Digit formation in some higher vertebrates is blocked by selective inhibitors of the effector pathway of apoptosis (Milligan *et al*, 1995, Jacobson *et al*, 1996). Apoptosis-deficient flies die early in development (White *et al*, 1994A) and mice in which one critical effector caspase (caspase 3) has been deleted by target gene disruption die perinatally with a vast excess of cells in their central nervous system (see also chapter 1.6.3.3.).

In humans defective apoptosis has been implicated in several diseases, including a variety of neurodegenerative diseases, immune system disorders and carcinogenesis (reviewed by Savill, 1994; Thompson, 1995, Haecker *et al*, 1995; Martin *et al*, 1995;

Nicholson, 1996). Diseases related to impaired apoptosis can be divided into disorders of excessive apoptosis and those where insufficient apoptosis occurs. Thus failure to regulate apoptosis negatively, resulting in too much cell death has been associated with degenerative diseases of the central nervous system such as Alzheimer's disease in which progressive and irreversible memory and cognitive loss appears to be the result of premature hippocampal neuronal death (Su *et al*, 1994; Lassmann *et al*, 1995; Smale *et al*, 1995) and clinical AIDS resulting from the gradual depletion of $CD4^+$ T cells during HIV infection (reviewed by Gougeon, 1996). Failure to regulate apoptosis positively, resulting in insufficient apoptosis, may be a component in some autoimmune diseases like lupus erythematosus, where a failure to complete apoptosis at the engulfment stage results in the recognition of cryptic epitopes, which are normally proteolytic cleaved during the apoptotic pathway, by lupus autoantibodies (Williams, 1994; Tan, 1994; Casciola-Rosen *et al*, 1994A; Casciola-Rosen *et al*, 1995). Failed apoptosis may also be a feature in carcinogenesis and may be the strategy of persistent and sustained viral infections (Evan *et al*, 1995; Osborne, 1995; White, 1995).

1.4. MOLECULAR REGULATION OF APOPTOSIS

In the past decade and especially in the past three years, it has become clear that apoptosis is controlled by a large number of genes. These seem to be evolutionarily conserved from simple eukaryotic organisms, through nematodes to mammals and some have also been adopted by viruses. First evidence of these genes came from studies on the nematode worm *Caenorhabditis elegans* (reviewed by Ellis *et al*, 1991, Driscoll, 1992; Miura *et al*, 1996; Hengartner, 1996). The nematode generates 1090 cells during its development of which 131 die by apoptosis. Each individual cell, programmed to die, does so at a certain time of development and each of the deaths can be followed by light microscopy, due to the transparency of the nematode worm. Genetic screening of viable nematode mutants which are deficient in apoptosis has led to the isolation of many alleles that affect the apoptotic pathway. So far 14 nematode genes have been found to be involved in developmental cell death. These genes have been placed in a developmental pathway of apoptosis, divided into

four distinct steps: determination/commitment, execution, engulfment and degradation.

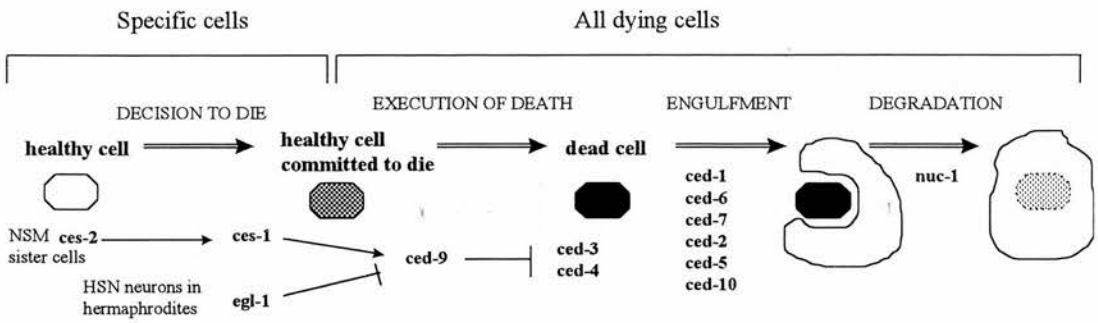


Figure 1.1. The genetic pathway of apoptosis in *C. elegans*. The proposed regulatory interactions between adjacent genes in the pathway are indicated: arrow, positive interaction; bar, negative interaction (adapted from Miura *et al*, 1996).

Three genes lie at the centre of the regulation of cell death in the nematode, namely *ced3*, *ced4* and *ced9*. CED3 is a caspase and effects death. It is the prototype of the cysteine protease family of which one mammalian member is the subject of this thesis. CED4 protein activates CED3 and CED9 inhibits this activation. All three genes are direct homologues of genes with similar function in mammals. In the following sections, the roles of these three gene families are described.

1.4.1. Regulation of the apoptotic pathway by CED9 and its mammalian counterparts

The *ced9* gene consists of a 1.3kb mRNA that is highly expressed *in C. elegans* embryos and encodes a negative regulator of developmental cell death (Hengartner *et al*, 1992). Gain of function mutations in the *ced9* gene prevent apoptosis in *C. elegans*, whereas loss-of-function mutations in *ced9* cause ectopic cell death that result in *C. elegans* embryonic lethality. *Ced9* is an element of a polycistronic locus that also contains the gene *cyt-1*, which encodes a protein similar to cytochrome b560 of complex II of the mitochondrial respiratory chain (Hengartner *et al*, 1994). *Ced9* encodes a protein of 280 amino acids that shares significant amino acid sequence similarity to the human Bcl-2 family of cell death regulators (reviewed

by Hale *et al*, 1996; Hermann *et al*, 1996; Park *et al*, 1996; Yang *et al*, 1996; Kroemer, 1997A; Kroemer *et al*, 1997B).

The *bcl-2* gene was the first member to be identified of the multigene *bcl-2* family. It was first detected as a putative oncogene located near the t(14;18)(q32;q21) translocation breakpoint common to many human follicular lymphomas and was first recognised as a survival factor without significant mitogenic activity by Vaux *et al*, 1988. Bcl-2 is widely expressed during early murine foetal development and in adults Bcl-2 expression is found mainly in tissues that are renewed from stem cells, are long lived, or have proliferative ability. Together with many other members of the Bcl-2 family, Bcl-2 is predominantly localised in the outer mitochondrial membrane, but is also present in the endoplasmic reticulum and the nuclear membrane. Overexpression of *bcl-2* protects from apoptosis by a variety of stimuli in many cell types and *bcl-2* expression in *C. elegans* can substitute for *ced9* loss of function mutations and suppress apoptosis. Ectopic or dysregulated expression of *bcl-2*, however, does not suppress all types of physiological cell death, including negative selection of thymocytes, lymphocytes and apoptosis by the Fas pathway following treatment with anti-Fas antibody (Chiu *et al*, 1995; Strasser *et al*, 1995; Sarfaz *et al*, 1995; An *et al*, 1996), which suggests that there are at least two pathways of apoptosis, one *bcl-2* dependent and another *bcl-2* independent.

In place of the single *C. elegans* negative regulator of apoptosis, CED9, mammalian apoptosis appears to be regulated by multiple genes. Protein homologues to Bcl-2 have been identified by a number of approaches, including coimmunoprecipitation with Bcl-2 and polymerase chain reaction (PCR) cloning, using degenerate primers. Evidence to the subcellular functions of individual Bcl-2 homologues from yeast two-hybrid assays and from structural analysis and has shown that the Bcl-2 family comprises both death-inducing and death-inhibitory members. Death antagonists include Bcl-2, Bcl-xl, Bcl-w, Bfl-1, Brag-1, Mcl-1 and A1 and death agonists include Bax, Bak, Bad, Bid, Bik, Hrk and Bcl-xs (an alternative RNA splice product to *bcl-xl* from the *bcl-x* transcript). Individual Bcl-2 family members differ in their tissue- and activation-dependent expression patterns, as well as in structural features.

Gene knock-out studies of individual Bcl-2 family members in mice support the theory that different members may be required for cell survival or deletion in particular tissues. Null mice of the death antagonist *bcl-2* or the agonist *bax* are viable and initially develop normally. Tissue and cell type specific abnormalities, due to defective cell survival of various cell types, become apparent only after birth and include thymocyte and B cell hyperplasia and male infertility. In contrast homozygous *bcl-x* knock-out mice show massive apoptosis in haematopoitic cells and central nervous system neurons. The homozygous null animals die at about embryonic day 13. These results suggest selective functions of the bcl-2 family members at certain times and in specific tissues, whilst at other times and situations there may be substantial functional redundancy.

Structural analysis has shown that most members, with the exception of Bid and Bad, possess a COOH-terminal transmembrane region and variable amounts of the Bcl-2 homology regions (BH1 to BH4). These homology regions together with the hydrophobic membrane domain determine the subcellular localisation and the capacity to interact with each other or unrelated proteins. Deletion of the transmembrane domain of various family members, including Bcl-2 and Bax, prevents targeting to the mitochondrial membrane and either abrogates or diminishes the function. Yeast two-hybrid assays and deletion mapping has shown selective formation of homo- and heterodimers among Bcl-2 family members, involving interactions in different BH regions, and a hierarchy in the strength of binding between various partners with the equilibrium state between these dimersations dictated in part by the relative abundance of each of the interacting proteins. Developmental cell death or survival in mammals might thus be regulated in a number of different ways and seems to be mediated, at least in part, by competitive dimerisation between selective pairs of antagonists and agonists forming a life-death rheostat. Which of the variety of homo- and heterodimers of family members that have been identified *in vitro* are true regulators of apoptosis *in vivo* is still largely unresolved. Some dimers may form part of regulatory, higher-order multiprotein complexes. Additional complexity is added by the finding that some members of the Bcl-2 family in this life-death rheostat might be common dimerisation partners, such as Bax, which in addition to Bcl-2 and Bcl-xl,

heterodimerises with MCL1 and A1. In contrast other family members heterodimerise selectively. Thus Bcl-xs only heterodimerises with Bcl-xl and Bcl-2. These observations suggests potential redundancy and a very wide range of possible death inhibitory or stimulatory events mediated by the Bcl-2 family. It should be noted, however, that most studies to date have not addressed the possibility that selective interactions may be critical at particular intracellular sites, or in certain cell types.

Expression levels and dimerisation, however, may not be the only parameters that determine the regulatory role of the Bcl-2 family members in apoptosis. Posttranslational modifications may have a major impact and add further to the complexity of regulation of apoptosis. Serine-phosphorylation has been shown to regulate the function of some individual members, which links the apoptotic regulatory pathway to signal transduction cascades. Inactivation by proteolysis may also be involved. Bcl-2 is highly susceptible to trypsin and chymotrypsin proteolysis, and apoptosis in HIV infected lymphocytes has been attributed to cleavage of Bcl-2 by the HIV protease.

The mechanism by which the complex Bcl-2 family regulates apoptosis is not yet clear, but as the role of mitochondria in apoptosis becomes further elucidated, the Bcl-2 family of apoptosis regulators emerge as an important link between mitochondria and apoptosis. Initial evidence for this link came from two independent studies. Bcl-2 was shown to be localised in the outer mitochondrial membrane (Reed, 1994) and cell-free apoptosis inducing extracts from *Xenopus* eggs were shown to require the presence of heavy membrane fractions which are rich in mitochondria (Newmeyer *et al*, 1994). Addition of Bcl-2 protein to those extracts blocks their capacity to initiate apoptosis suggesting a direct link between mitochondrial apoptotic signalling and negative regulation by Bcl-2. Pharmacological and functional studies have since revealed that mitochondrial alteration appears to play a central role in the apoptotic process and suggest that apoptosis might be triggered by a disruption of the mitochondrial membrane function. The critical observations in this currently confused area appear to be as follows (reviewed by Martins *et al*, 1997A; Kroemer *et al*, 1997B). First, the mitochondrial membrane potential dissipates in apoptosis and, at least in some systems, appears to be a critical factor in triggering these terminal

effector events (see also 1.6.5.). Second, Bcl-2 and its close structural homologue Bcl-x are structurally reminiscent of bacterial pore-forming proteins and are thus capable of regulating the flux of ions and larger molecules across the outer mitochondrial membrane. Third, mitochondria, being the intracellular location of the terminal respiratory chain enzymes, are promiscuous sites for the sensing of alterations in cellular redox equilibrium or generation of reactive oxygen species. Finally, certain additional molecules, amongst them the electron transfer protein cytochrome c (Liu *et al*, 1996) and the mitochondrial protease AIF (apoptosis inducing factor) (Susin *et al*, 1996) are released from mitochondria and are essential for activation of apoptosis (see also 1.6.5.). It is not too difficult to link these observations into a single mechanism wherein mitochondrial membrane changes, induced by reactive oxygen species damage or initiated by modification of Bcl-2 or Bcl-xl function, lead to the release of sequestered caspase activators. The details of this putative mechanism are still unproven. Nor is it completely clear whether mitochondrial depolarisation plays an initiating or secondary role.

Recent advances have identified a novel cytosolic proteins Apaf-1 (apoptotic protease activating factor) as the potential homologue of the *C. elegans* death gene *ced4* (Zou *et al*, 1997). A critical role of Bcl-2 and other antiapoptotic family members appears to be the inactivation of the linker molecule CED4.

1.4.2. Regulation of the apoptotic pathway by CED4 and its mammalian counterpart

Like CED9 and CED3 (see chapter 1.6.), CED4 is required for developmental cell death in *C. elegans* (Yuan *et al*, 1992; reviewed by Miura *et al*, 1996). The *ced4* gene is most abundantly expressed during embryogenesis. *Ced4* encodes a 549 amino acid protein, which is very hydrophilic and contains a structural motif which resembles a calcium binding domain. *Ced4* loss of function mutant animals are structurally normal despite a general lack of apoptosis and a 30% slower growth rate than wild-type animals. Overexpression of the *ced4* gene causes cells that normally survive to undergo apoptosis and CED4 thus acts as an activator of developmental cell death. CED4 activity in *C. elegans* seems to be negatively regulated by CED9, as mutations in *ced4* can suppress the embryonic lethality of *ced9* lack-of -function mutation. Two splice variants of *ced4* have been identified: *ced4s* and *ced4l* which exhibit different

functions (Shaham *et al*, 1996). Recent studies in yeast and mammalian cells show that CED4L is a cytosolic protein which binds directly and simultaneously to CED9 and CED3 or to the mammalian homologous Bcl-xl and caspases (Spector *et al*, 1997; Irmler *et al*, 1997A; Chinnayan *et al*, 1997; Wu *et al*, 1997A; Wu *et al*, 1997B). Interaction of CED4 with CED3 seems to be through a novel protein-protein interaction motif named CARD (caspase-recruitment domain). Thus CED4 could provide the direct link between CED9 and CED3. The multiple interactions suggest a mechanism by which CED9 through interaction with CED4 could inhibit the ability of CED4 to activate CED3. Membrane localisation may be important for this regulation of CED4 activity as coexpression of *ced4* with *ced9* targets CED4 to intracellular membranes (Wu *et al*, 1997A; Wu *et al*, 1997B; Spector *et al*, 1997; Chinnaiyan *et al*, 1997; Irmler *et al*, 1997A). The recent identification of Apaf-1, a cytoplasmic 130kDa human protein with high sequence similarity to CED4 and to caspase 3 adds further complexity to the regulatory function of CED4 (Zou *et al*, 1997). Apaf-1 contains multiple protein-protein interaction motifs, binds cytochrome c and triggers the activation of caspase 3 *in vitro* in the presence of both cytochrome c and ATP. This suggests a model in which the Bcl-2 family of proteins by controlling the release of cytochrome c might indirectly regulate the cytochrome c dependent Apaf-1 function of caspases activation (see also chapter 1.6.5.). The regulatory role of CED4 and its mammalian counterpart(s) is thus only beginning be understood but it is clear that a major role is to activate the caspases, one of which is the theme of the experiments of this thesis. Before considering these in detail, however, it is important to discuss how the terminal effector events of apoptosis and other immediate regulatory mechanisms are influenced by incoming stimuli of both physiological and pathological nature.

1.5. MOLECULAR INDUCERS OF APOPTOSIS

The regulatory mechanisms described in the preceding section are present in many cell types and the terminal, caspase-dependant pathway also appears to be common to cells of many lineages resulting from a wide variety of stimuli. The transduction of these stimuli, however, so as to activate these common terminal events takes an enormous variety of routes, too diverse to be discussed in detail here and may depend on the cell type and its characteristic gene expression, the cellular environment, the state of differentiation, the cell cycle-phase and the dose of the reagent

In principle, certain generalities about the transduction of apoptotic stimuli are already apparent. First, both physiological and injury-related stimuli initiate apoptosis under the appropriate circumstances. Amongst the known injury-related stimuli are those arising within the nucleus (DNA damage), mitochondria (reactive oxygen species) and cell membrane (ceramide) and it is clear that other forms of injury may also trigger apoptosis.

Site of perturbation	*Mechanism of perturbation*
Nucleus	Perturbation of DNA integrity Directly: DNA damage Indirectly: Inhibition of nuclear enzymes Alteration of gene expression
Cell surface	Activation of cell membrane receptors Disruption of regulation of plasma membrane solute flux
Cytosol and organellar compartments	Membrane perturbation Alteration of microtubule organisation Disruption of actin network Disruption of the cytosolic milieu Disruption of intracellular membrane ion gradients Alteration of protein synthesis Alteration of protein phosphorylation Alteration of signal transduction

Table 1.1. Sites and mechanisms of apoptotic induction (adapted from Wertz *et al*, 1996).

Physiological stimuli include those resulting from binding of specific cytokines to their receptors on the cell surface (Fas, tumour necrosis factor receptor (TNFR) and other members of the TNF receptor superfamily such as death receptors DR3, 4 and DR5) which can activate the terminal pathway by non-transcriptional means. There are also more subtle stimuli, most still rather poorly defined, that regulate transcriptional activation before the terminal effector pathway is affected, such as in apoptosis induced by transcriptional activation of c-myc and nur77. A variety of oncogene transcripts also modify the susceptibility to apoptosis (ras, src and abl) (reviewed by Hoffman *et al*, 1994; White, 1994B; Evan *et al*, 1995; Hale *et al*, 1996; Miura *et al*, 1996). A further important mechanism in the induction of apoptosis is the activation of the terminal pathway by specific enzymes transferred from adjacent sites. This is one part of the mechanism of the killing by CTL, through transfer of the caspase activating serine protease granzyme B. Finally there are emerging mechanisms that appear to link these various transduction pathways together. Thus activation of the TNFR may initiate non-transcriptional activation of caspases, and may also by a transcriptional mechanism via NF_kB activation inhibit apoptosis. Further, some of the pathways described may be at least partially dependent on others. Thus apoptosis induced by c-myc or p53 is dependent on the activity of the Fas signalling system (White, 1996A).

In this thesis experiments are described in which apotosis is induced by three separate mechanisms- nuclear DNA double strand breaks (through action of the chemotherapeutic drug etoposide), inhibition of protein kinase C (through treatment with staurosporine) and stimulation of the Fas signalling pathway.

1.5.1. Reagents acting within the nucleus: etoposide

Etoposide, an antineoplastic drug, induces apoptosis by formation of double strand DNA breaks through inhibition of topoisomerase II (reviewed by Liu, 1989; Froelich-Ammon *et al*, 1995; Capranico *et al*, 1997). Topoisomerase II binds to DNA covalently and forms transient single- or double-strand DNA breaks to modulate DNA topology. Etoposide binds to topoisomerase II and stabilises these covalent topoisomerase II DNA cleavage complexes and hinders the reclosure of the DNA breaks. These transient protein-associated breaks are disrupted and are converted into

permanent double-strand breaks, no longer held to together by proteinaceous bridges, following the traverse of replication complexes or helicases. DNA damage initiated by etoposide treatment leads to activation and change in the stability of the tumour suppressor gene p53. Stabilised p53 activates the transcription of target genes and induces growth arrest or, alternatively, induces apoptosis in many cell types. Etoposide is thus commonly used for the induction apoptosis and the study of the role of caspases in a variety of cell culture systems including human HL60, U937 and ML-1 leukaemia cells and THP.1 monocytic tumour cells (Kaufmann, 1989; Martins *et al*, 1997; Kaufmann *et al*, 1993; MacFarlane *et al*, 1997; Mashima *et al*, 1995A; Mashima *et al*, 1995B; Morana *et al*, 1996).

1.5.2. Reagents acting in the cytoplasm: staurosporine

Staurosporine, a microbial alkaloid extracted from streptomyces species, is a nonselective protein serine/threonine kinase inhibitor. Staurosporine induces apoptosis in many normal and transformed cells in cell culture, including human leukaemia cell lines such as HL-60 and MOLT-4, Jurkat T cells and human fibroblasts (Bertrand *et al*, 1994; Orth *et al*, 1996A, Na *et al*, 1996; Jacobson *et al*, 1993; Jacobson *et al*, 1996). Staurosporine prevents protein phosphorylation by multiple kinases, which include protein kinase C, protein kinase A, Ca^{2+}/calmodulin dependent kinase II and tyrosine kinases. It has been proposed to act at high (micromolar) concentrations by blocking the intracellular signals initiated by extracellular survival factors, which are often transmitted by protein phosphorylation of one or several cytoplasmic factors (Herbert *et al*, 1990; Yanagihara *et al*, 1991; Bertrand *et al*, 1994). Ongoing activation of certain protein kinases, possibly protein kinase C or tyrosine kinases, thus seems to be involved in maintaining the survival of staurosporine sensitive cells and their inhibition may mimic a state of factor depletion. Apoptosis induced by staurosporine can be blocked by Bcl-2 or Bcl-xl (Orth *et al*, 1996; Chinnaiyan *et al*, 1996A).

1.5.3. Reagents acting on the cell surface: Receptor ligands

1.5.3.1. Fas/CD95 apoptotic pathway

Fas-induced apoptosis is currently one of the best-characterised apoptotic pathways (reviewed by Nagata and Golstein, 1995; Nagata, 1996; Yuan , 1997;

Nagata, 1997). Human Fas/CD95 is a type I membrane protein which consists of 325 amino acids with an NH_2-terminal signal sequence, a central membrane spanning domain and a COOH-terminal cytoplasmic region which carries a conserved domain of about 80 amino acids, designated the death domain. It belongs to the tumour necrosis factor (TNF) and nerve growth factor (NGF) receptor family and is the receptor for Fas ligand (FasL) (Smith *et al*, 1994). Fas is expressed in many tissues including the thymus, liver, heart and kidney, while FasL appears to be predominantly expressed in activated T-lymphocytes, natural killer cells and immunoprivileged tissues such as the testis and the eye (Trauth *et al*, 1989; Vignaux *et al*, 1995; Anel *et al*, 1994).

Fas-mediated apoptosis occurs in the presence of inhibitors of protein and RNA synthesis and also in enucleated cells, indicating that no specific gene induction is required (Yonehara *et al*, 1989; Itoh *et al*, 1991; Schulze-Osthoff *et al*, 1994). Fas-mediated apoptosis is involved in the down-regulation of immune reactions and T-cell mediated cytotoxicity and has been implied in cytotoxic T-cell mediated diseases, including liver destruction in fulminat hepatitis, lymphopenia in acquired immunodeficiency (AIDS) and the destruction of pancreas in insulitis. Genetic mutations in murine Fas (lpr mutation) or FasL (gld mutation) lead to defective T cell receptor-induced cell death of mature T cells, resulting in autoimmune disease that resembles human systemic lupus erythematosus. A human dominant-negative Fas mutation leads to autoimmune lymphoproliferative syndrome (Fisher *et al*, 1995).

Signalling by Fas requires the cross-linking of Fas achieved physiologically by binding the endogenous FasL, but mimicked by some anti-Fas antibodies (Dhein *et al*, 1992; Suda *et al*, 1994). Receptor engagement with trimerised FasL or aggregated antibody (IgM class monoclonal Fas antibody or IgG3 class APO1 antibody) induces trimerisation of Fas and the trimerised cytoplasmic region transduces the signal (Itoh *et al*, 1993, Tanaka *et al*, 1997). Within seconds after receptor oligomerisation a death domain containing adapter, called FADD/MORT1 (Fas-associating protein with death domain/mediator of receptor induced toxicity 1) binds to trimerised Fas via interactions between the death domains (Boldin *et al*, 1995; Chinnaiyan *et al*, 1995; Chinnaiyan *et al*, 1996B; Kischkel *et al*, 1995). The death effector domain of

FADD/MORT1 then binds to corresponding sequence motifs within the prodomain of caspase 8/FLICE/MACH and caspase 10/Mch4/FLICE2 which are members of the effector proteases, which form the main theme of the experiments in this thesis (Boldin *et al*, 1996; Muzio *et al*, 1996; Fernandes-Alnemri *et al*, 1996; Vinzenz *et al*, 1997).

How the recruitment of caspase 8 and 10 to the Fas-receptor complex results in the sequential activation of a caspase cascade is not clear (see also chapter 1.6.5.). One possibility is that interaction of caspase 8 or 10 with FADD/MORT1 may result in oligomerisation and autocatalytic activation of the caspases which could initiate proteolytic activation of downstream caspases (Muzio *et al*, 1997). Other FADD interacting molecules have been identified, however, suggesting that FADD coupling to downstream caspases may be indirect or sequential. Amongst these interacting molecules is a novel member variously called FLIP (FLICE-inhibitory protein)/FLAME-1 (FADD-like antiapoptotic molecule)/CASH (caspase homologue)/Casper (caspase 8 related protein)/I-FLICE (inhibitor of FLICE) (Srinivasula *et al*, 1997; Goltsvec *et al*, 1997; Shu *et al*, 1997; Irmler *et al*, 1997B). This protein exists in at least six different splice variants with apparently opposing functions. Different splice variants are recruited to the Fas signalling pathway through interaction with FADD and can inhibit or induce both Fas- and TNF-induced apoptosis. Even though not a caspase itself it contains two NH_2-terminal death domains and also contains a caspase domain homology region with high sequence similarity to caspase 3, caspase 8 and caspase 10. It can therefore interact with all three caspases and can most likely recruit all of those caspases through FADD to the Fas/TNF signalling complex.

A variety of models can be built around how FLIP might negatively or positively regulate apoptosis. One possibility is that it might interfere with the assembly of a functional death receptor signalling complex. An intriguing possibility is that splice variants with apoptotic functions might constitute part of an active caspase molecule, another possibility is that FLIP, which can simultaneously bind caspase 8 and caspase 3, may simply recruit procaspase 3 to the vicinity of caspase 8, where it can be activated (see chapter 1.6.5.). The importance of the mammalian FLIP molecule in the

control of apoptosis is further supported by the identification of viral protein homologues (Hu *et al*, 1997; Thome *et al*, 1997; Bertin *et al*, 1997).

Other Fas pathway interacting molecules, including the ubiquitin-conjugating enzyme UBC9 (Becker *et al*, 1997), have been identified. Their function in the Fas-mediated apoptosis is, however, less clear.

1.5.3.2. Tumour necrosis factor (TNF) induced apoptosis

Tumour necrosis factor (TNF), like FasL, is a cytokine in the TNF family. It binds to two receptors (TNFR1 and TNFR2) (reviewed by Tewari *et al*, 1996; Nagata, 1997). While the main activity of FasL is to trigger cell death, TNF affects the growth, differentiation and function of multiple cell types and is a mediator for inflammation, cellular immune responses as well as apoptosis. Signalling by TNFRs requires cross-linking of TNFRs and can be achieved experimentally with monoclonal antibodies to TNFRs, with membrane bound TNF or with purified, soluble TNF. The functional form of soluble TNF exits as a trimer (Tanaka *et al*, 1997) which upon binding to TNFR1 induces trimerisation of this receptor. The trimerised cytoplasmic region of the receptor includes a death domain essential for transduction of the signal, leading to apoptosis (reviewed by Eder, 1997). Apoptosis induced by TNFR1 trimerization occurs via the death domain-containing protein TRADD (TNFR1-associated death domain protein) which binds to TNFR1 (Hsu *et al*, 1995). The death domain of TRADD then binds to FADD/MORT1 and shares the signal downstream machinery with Fas signalling recruiting caspase 3, caspase 8 and caspase 10. Alternatively TRADD binds via interactions of their death domains to RIP (receptor interacting protein), which is a serine threonine kinase (Stanger *et al*, 1995). RIP is also recruited by DR-3/Wsl-1 receptor, a recently identified new member of the TNFR family, and overexpression of RIP results in the induction of apoptosis (Chinnaiyan *et al*, 1996C). RIP itself, however, does not contain a death effector domain and may thus trigger another apoptotic pathway through the recruitment of another molecule. A potential candidate is the recently identified death domain containing protein RAIDD/CRADD (RIP-associated ICH-1/CED3 homologous protein with death domain/caspase and RIP adapter with death domain) (Duan *et al*, 1997; Ahmad *et al*, 1997A). RAIDD/CRADD binds RIP through its COOH terminal

death domain and recruits RIP to caspase 2 (ICH-1) with a caspase 2 NH_2-terminal homology domain. Although a direct connection between RAIDD/CRADD and DR3/Wsl-1 and TNFR1 has not been demonstrated RAIDD/CRADD presumably functions like FADD and might transduce the apoptotic signals from Fas/TNF to the caspase effector cascade through interaction with procaspase 2 (ICH-1). Apoptosis induced by both TNF and Fas is inhibitable by the cowpox virus encoded serpin CrmA and the recently identified novel family of viral death effector domain-containing molecules (Hu *et al*, 1997; Bertin *et al*, 1997; Thome *et al*, 1997).

In summary, the known elements in the Fas and TNF signalling pathway point to the possibility of a variety of outcomes depending on the circumstances of cytokine signalling. Although Fas and TNFR share the effector pathway via FADD, TNFR1 stimulation also activates potential death-inhibitory transcription-dependent mechanisms through $NF_\kappa B$ and specific activation of caspases 3, 8 and 10 may be effected through FADD, whilst activation of caspase 2 (ICH-1) may be through RAIDD/CRADD.

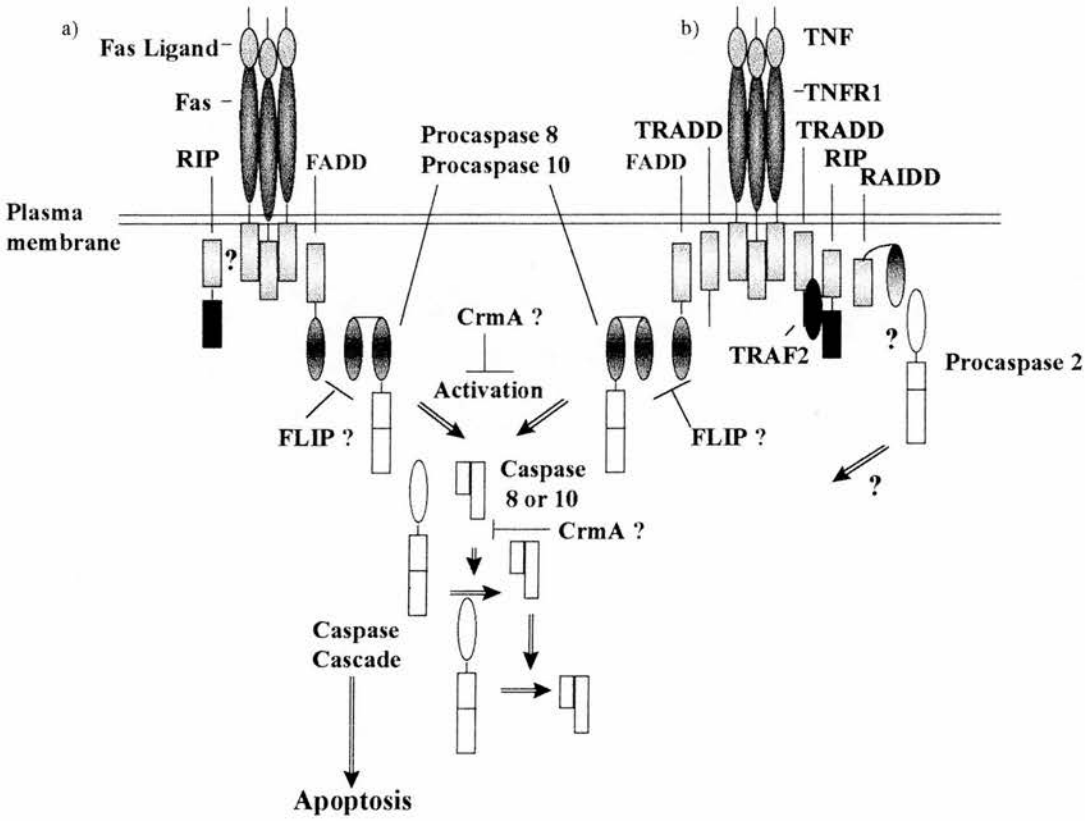


Figure 1.2. Possible interactions of the components of Fas and TNF apoptotic signalling pathway. A) Fas-induced apoptosis, b), TNF-induced apoptosis. Caspase 8 and 10 acting as a linkage between the signalling complexes and the caspase family.

1.6. THE EFFECTOR PATHWAY OF APOPTOSIS: THE CASPASE FAMILY

1.6.1 Discovery of the family of ICE-like proteases/caspases

Current appreciation of the significance of caspases as effectors of apoptosis originated from the identification and cloning of the *C. elegans ced-3* gene (Yuan *et al*, 1990; Yuan *et al*, 1993). *Ced-3* encodes a cytoplasmic cysteine protease of 503 amino acids whose proteolytic activity is absolutely required for the execution of apoptosis in nematode development (Xue *et al*, 1996; Hugunin *et al*, 1996). CED3 shares 29% amino acid identity with the human interleukin-1β converting enzyme ICE/caspase 1 (Yuan *et al*, 1993). Structurally unrelated to any other known class of proteases, CED3 and caspase 1/ICE are the founder members of a family of proteases, named caspases in reference to their catalytic mechanism as cysteine proteases with a cleavage specificity at aspartic acid residues (Alnemri *et al*, 1996). To date known caspase family members include caspase 1/ICE (Thornberry *et al*, 1992; Miura *et al*, 1993), caspase 2/ICH-1/NEDD-2 (Wang *et al*, 1994; Kumar *et al*, 1994), caspase 3/CPP32/Yama/apopain (Fernandes-Alnemri *et al*, 1994; Tewari *et al*, 1995; Nicholson *et al*, 1995), caspase 4/TX/ICH-2/ICErelII/Mih1 (Faucheu *et al*, 1995; Kamens *et al*, 1995; Munday *et al*, 1995, Alnemri, 1997), caspase 5/ICErelIII/TY (Munday *et al*, 1995; Faucheu *et al*, 1996), caspase 6/Mch2 (Fernandes-Alnemri *et al*, 1995A), caspase 7/Mch3/ICE-LAP3/CMH-1 (Fernandes-Alnemri *et al*, 1995B; Duan *et al*, 1996; Lippke *et al*, 1996), caspase 8/FLICE/MACH/Mch5 (Muzio *et al*, 1996; Boldin *et al*, 1996; Fernandes-Alnemri *et al*, 1996A), caspase 9/ICE-LAP6/Mch6 (Duan *et al*, 1996; Alnemri, 1997) and caspase 10/Mch4/FLICE2 (Fernandes-Alnemri *et al*, 1996B; Vincenz *et al*, 1997). Phylogenetic analysis of the known caspase family has led to their subclassification into two broad subfamilies: the caspase 1/ICE subfamily and the caspase 3/CPP32 subfamily.

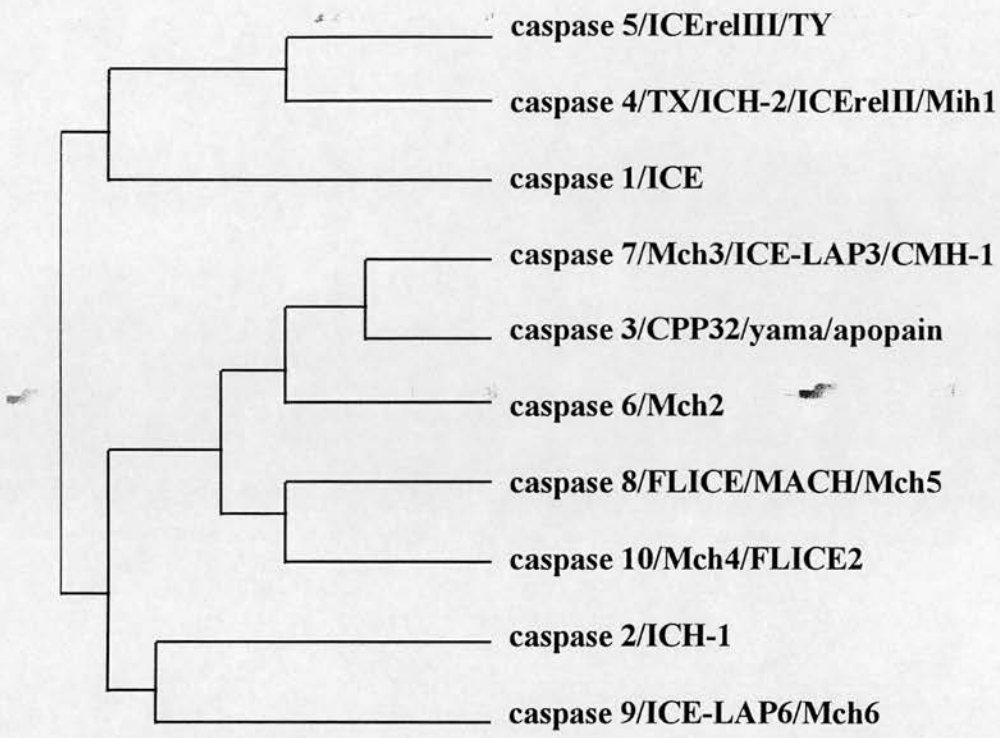


Figure 13. Phylogenetic tree of the caspase family

ICE/caspase 1 was the first mammalian member of the caspase family to be identified and much of the initial data on caspases was obtained from studies of caspase 1. It was initially discovered because of its activity as an activator of the cytokine interleukin-1β (IL-1β) in inflammation and was purified from the cytosol of the human monocytic cell line THP.1 (Thornberry *et al*, 1992). Caspase 1/ICE cleaves the inactive 33kDa IL-1β precursor at Asp116-Ala117 to generate the 17kDa fully active cytokine IL-1β.

1.6.1.1. Discovery of caspase 2: First hint of multiple CED3 homologues

In search for genes that are expressed during the development of the mammalian central nervous system a cDNA library, prepared from mouse neuronal precursor cells (NPCs) and isolated from the neuronal tube at embryonic day ten, was probed with mRNA isolated from postnatal and adult brain. Ten independent clones were isolated and named *Nedd* for NPC-expressed, developmentally downregulated (Kumar *et al*, 1992). Subsequent characterisation of those mouse genes showed that one gene, *Nedd2* encodes a protein similar to the mammalian enzyme caspase 1/ICE

and the *C. elegans* CED3 protein (Kumar *et al*, 1994). The human *Nedd2* gene, which was renamed *ICH-1* (*ICE* and *ced3* homologue)/caspase 2, was isolated from a human foetal brain cDNA library (Wang *et al*, 1994). *ICH-1* mRNA is alternatively spliced into two different forms. One mRNA species encodes a protein product of 435 amino acids, named *ICH-1*$_L$ which when overexpressed in Rat-1 cells induces apoptosis. The other mRNA encodes a 312 amino acid truncated version of ICH-1$_L$, named ICH-1$_S$, which terminates 32 amino acids after the pentapeptide QACRG of ICH-1L. Overexpression of ICH-1S suppresses Rat-1 cell death induced by serum deprivation.

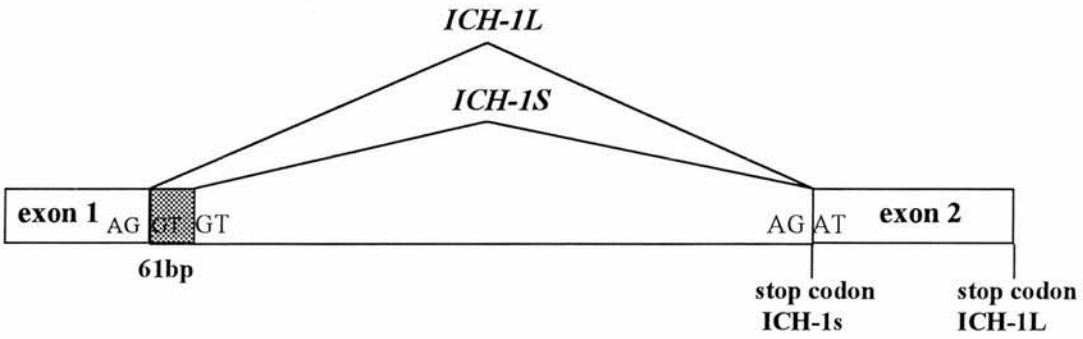


Figure 1.4. Alternative splicing of the *caspase 2/ICH-1* mRNA. The exons are shown in bars. The introns are shown in a line. Nucleotides at the intron/exon borders are marked. The positions of the ICH-1S and ICH-1L stop codons are indicated.

Nothern blot analysis of human foetal heart, brain, lung, liver and kidney, with a probe that hybridises to both *ICH-1*$_L$ and *ICH-1*$_S$, show low levels of expression. An *ICH-1*$_S$ specific probe shows enhanced expression in the embryonic brain than in the lung, liver and kidney. Northern blot analysis of adult tissues shows detectable levels in all tissues examined with higher levels of expression in placenta, lung, kidney and pancreas than in heart brain and skeletal muscle. Chromosome mapping places both *ICH-1*$_L$ and *ICH-1*$_S$ sequences on chromosome 7q35 (Tiso *et al*, 1996).

1.6.2. Structure and composition of the caspases: Caspase 1/ICE as the caspase prototype

Caspase 1/ICE has an open reading frame of 1212 bp encoding a proenzyme of 404 amino acids with a predicted molecular weight of 45kDa. The mature and

active form of caspase 1 is derived from this 45kDa precursor by proteolytic cleavage at Asp-X bonds where X is Ser104, Asn120, Ser298 andAla317. This results in the proteolytic removal of the NH_2-terminal 119 amino acids and an internal fragment spanning residues 289-316 and the formation of two subunits of 10kDa (p10) and 20kDa (p20) molecular weight. Two p10 and two p20 caspase 1 subunits heterodimerise to form the tetrameric active enzyme. (Thornberry *et al*, 1992; Wilson *et al*, 1994, Walker *et al*, 1994). This oligomerisation occurs by a fairly ill-defined process most likely involving p10 and p20 subunits from different p45 precursor proteins (Gu *et al*, 1995A). Caspase 1 and caspase 3 are so far the only family members with known crystal structure. Global fold, topology and quaternary structure of both enzymes is unlike that of proteases of other classes (Wilson *et al*, 1994, Walker *et al*, 1994; Rotonda *et al*, 1996).

Sequence comparisons between the caspase family members show approximately 30% overall amino acid identity, with a high degree of homology in the domains that make up the mature proteases, particularly around the active site. For some of the caspases the exact subunit sizes of the active enzyme are still undetermined. Molecular masses, however, have been predicted and together with sequence comparison suggest that all caspase members are synthesised as inactive proenzymes, processed exclusively at Asp residues and, in most cases, before small amino acids such as Gly, Ala or Ser to the active enzyme. Differences between individual caspases exist in the overall size of the proenzyme, the size of the prodomain and the size of the active subunits. The proenzyme of caspase 2 (ICH-1) (the main subject of this thesis) has a predicted molecular weight of 48kDa, with subunits of 12kDa and 18kDa, a 1.4kDa linker peptide and a large prodomain of 16.6kDa (Xue *et al*, 1996). In comparison, caspase 3 is synthesised as a 32kDa proenzyme cleaved into 12kDa and 17kDa subunits with a small 3kDa prodomain and no linker region (Nicholson *et al*, 1995). In addition to the differences in overall size between individual caspase family members, the prodomains show, in comparison to the amino acid sequences that make up the active proteases, a broader divergence in the amino acid sequence, suggesting that these domains contain additional information and may be responsible for the functional differences between the family members. One obvious example is the presence of the death domains within

the prodomain of caspase 2, 8 and 10 permitting their interaction with linker proteins in the Fas and TNFR pathways (see chapters 1.5.3. and 1.6.5.).

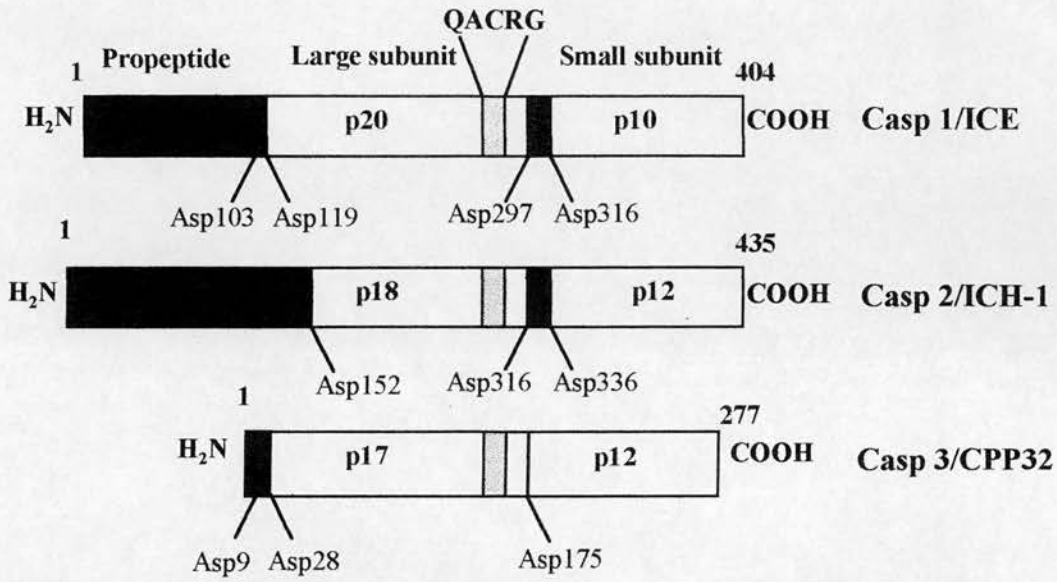


Figure 1.5. Subunit composition of three caspase family members. Diagram of the protease subunits and their relationship to the protease precursors. The size and the position of each protease subunit is depicted within the protease precursor. The position of each active site pentapeptide (QACRG) is indicated by a shaded box. Angled lines underneath indicate proteolytic cleavage sites and are numbered according to their position in the protease precursor.

The catalytic mechanism and the substrate specificity of caspase 1 have been revealed by the use of synthetic oligopeptide inhibitors. Synthetic substrates in the form of small peptides (usually four amino acids) are engineered to mimic the proteolytic substrate cleavage site of the enzyme and contain the amino acid residues essential for catalytic recognition. They are synthesised as peptide aldehydes or chloromethylketones and act as competitive inhibitors that undergo nucleophilic addition of the catalytic cysteine to form thiohemiacetals and thiohemiketals (Thornberry *et al*, 1995). The synthetic tetrapeptide acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO) inhibits the catalytic mechanism of caspase 1 by binding covalently to the sulphur atom of Cys285 (Thornberry *et al*, 1992). Recent advances in the design of peptide inhibitors for individual caspase family members has led to the identification of more specific peptide recognition motifs for caspase 1 and other family members (Thornberry *et al*, 1997) (see chapter 1.6.3.2.). Ac-WEHD-CHO is

thus the preferred recognition motif for caspase 1 and is also the most potent reversible, small molecule inhibitor for any caspase.

The inhibition profile of the caspase 1 activity by the peptide inhibitor together with results from site-directed mutagenesis led to the classification of caspase 1 as a cysteine protease with substrate specificity for an aspartic acid residue at the P1 position (the first amino acid NH_2- terminal to the cleavage site). The catalytic cysteine is located in the p20 subunit at position 285. The active site, however, is formed by amino acids from both subunits. Enzyme catalysis requires p20 subunit amino acids residues His237 and Gly238. Critical elements in the formation of the binding pocket for the carboxylate side chain of P1 Asp are p20 amino acids Arg179, Gln283 together with p10 residues Arg341 and possibly Ser347 (Wilson *et al*, 1994; Walker *et al*, 1994). These important amino acid residues for recognition of P1 Asp and for catalysis of caspase 1 are conserved in every member of the caspase family, together with a pentapeptide QACRG, containing the active cysteine (with the exception of caspase 8, caspase 9 and caspase 10 which have an R to Q substitution in the QACRG pentapeptide active site motif (Duan *et al*, 1996; Fernandes-Alnemri *et al*, 1996A; Muzio *et al*, 1996; Vincenz *et al*, 1997)). This suggests that all caspases members share the same catalytic mechanism and explains similar peptide preferences for individual caspases. There is more diversity, however, in the amino acids that putatively line the groove where the P2-P4 residues of the substrate lie, corresponding in caspase 1 to Val338, Trp340, His342, Pro343, Arg383 and Gln385. These amino acids are most likely responsible for the different macromolecular specificities and different biological functions amongst the various family members.

1.6.3. Involvement of the caspases in apoptosis

Definite proof that a particular protein contributes to the effector pathway of apoptosis requires that it is expressed in cells competent to activate the pathway, that overexpression initiates death and that downregulation or specific inhibition block cell death. At present only a few of the caspases fulfil all these requirements. Caspase genes are present in both vertebrates and invertebrates, and expression of many procaspase appears to be a feature of cells of most if not all lineages. Furthermore apoptosis has been induced in a variety of mammalian and insect cells following

overexpression of caspase 1 and most of the other members of the caspase family (Miura *et al*, 1993; Ahmad *et al*, 1997B; Song *et al*, 1997; Fraser *et al*, 1997A). This evidence alone is insufficient proof of a specific role of those caspases in cell death, however, as introduction of other known proteases of entirely different specificities has been claimed to induce death with many of the characteristic morphological features of apoptosis including membrane blebbing, chromatin condensation and DNA fragmentation (Williams *et al*, 1994). Evidence for the physiological role of caspases in apoptosis derives from the efficacy of specific protease inhibitors (natural or synthetic), the biology of genetically altered ('knock-out') mice, and protease activity in apoptotic cell extracts. In brief, the inhibitors, which can be shown to interfere with caspase protease function, when applied to cultured cells, produce retardation or in some cases complete abrogation of apoptosis. The mice lacking caspases as a result of germ-line gene knock-out sometimes lack apoptosis in certain lineages or in response to particular stimuli. And the cell-free extracts engage caspase activity which reproduces the effects of apoptosis on critical intracellular structures, such as the nuclear envelope, nuclear chromatin, and cytosolic, nuclear and cytoskeletal proteins. Some detail is given in the following paragraphs of these three strands of evidence for the physiological role of caspases in apoptosis.

1.6.3.1. Natural inhibitors

Naturally occurring inhibitors of caspases have been identified in both the mammalian and viral genome and functionally equivalent molecules are most likely to be encoded in the genome of insects. Viral inhibitors of caspases include the cowpox virus serpin CrmA, baculovirus proteins p35 and IAP and the recently discovered six viral FLICE inhibitory proteins (vFLIPs) including human molluscum contagiosum poxvirus (MCV) proteins MC159 and MC160, the equine herpesvirus-2 (EHV-2) protein E8, Kaposi's-sarcoma-associated human herpesvirus-8 protein K13, bovine-herpesvirus-4-encoded protein E1.1 and herpesvirus saimiri (HVS) protein.

The cowpox virus serpin gene, which encodes a 38kDa protein, known as the cytokine response modifier CrmA, has been shown to suppress caspase 1 induced apoptosis *in vitro* and inhibit the cleavage of IL-1β (Miura *et al*, 1993; Ray *et al*, 1992; Komiyama *et al*, 1994; Howard *et al*, 1995). CrmA cDNA transfected into cells

can inhibit apoptosis by nerve growth factor depletion (Gagliardini *et al*, 1994), isolation from contact with extracellular matrix (Bourdeau *et al*, 1995), Fas ligation (Tewari *et al*, 1995A; Tewari *et al*, 1995B; Enari *et al*, 1995A; Enari *et al*, 1995B; Los *et al*, 1995) and activation of the tumour necrosis factor receptor (Miura *et al*, 1995; Tewari *et al*, 1995C; Tewari *et al*, 1995D). In cell free extracts (see 1.6.3.4.) CrmA has been shown to inhibit the nuclear structural changes of apoptosis and the cleavage of the caspase substrate lamin A (Takahashi *et al*, 1996A). Hence the death pathway in all these systems converges onto a CrmA-inhibitable step. Successful inhibition of certain caspases appears, however, to require high levels of expression of the *CrmA* gene, not necessarily achieved during viral infection. Comparison of the kinetics of CrmA interaction with caspase 1, 3, 6, 7 and 8 have shown that these five caspases are inhibited with rates and affinities ranging over five orders of magnitude. CrmA shows the highest affinity for caspase 1 and the second highest for caspase 8, whereas caspase 7 is not inhibited by CrmA (Zhou *et al*, 1997).

Two baculovirus proteins, IAP and p35, can prevent insect cells from dying in response to infection. P35 blocks apoptosis in baculovirus infected insect cells, resulting in increased virus production (Crook *et al*, 1993; Clem *et al*, 1994) and can block cell death in several species including nematode, insect and mammalian cells (White *et al*, 1996B; Sugimoto *et al*, 1994; Hay *et al*, 1994). In mammalian cells overexpression of p35 inhibits proteolytic activity of caspase 1, blocks neuronal cell death induced by nerve growth factor depletion (Martinou *et al*, 1995; Rabizadeh *et al*, 1993) and blocks both TNF and FasL induced apoptosis (Beidler *et al*, 1995). Purified p35 has been shown to be a potent inhibitor of various other caspases including caspase 2, 3 and 4, and *C. elegans* CED3 (Bump *et al*, 1995). This antiapoptotic activity involves both cleavage by and inhibition of caspase family members (Bertin *et al*, 1996). Both p35 and CrmA possess internal cleavage sites for caspase family members. Cleavage at these sites results in a conformational change and a stable complex formation between p35 and the protease. Exchanging the cleavage site of CrmA to that of p35 confers the ability of p35 to arrest apoptosis in *C. elegans* embryos upon CrmA (Xue *et al*, 1995). Further clues to the caspase target(s) in mammals come from the recently identified *Spodoptera frugiperda*

caspase 1. This insect caspase has a similar sequence to mammalian caspase 3 and 7, has been shown to be capable of cleaving p35 and is potently inhibited by p35 (Ahmad *et al*, 1997B). Another baculovirus protein IAP (inhibitor-of-apoptosis) inhibits apoptosis induced by caspase 1 and caspase 2 overexpression (Hawkins *et al*, 1996) and directly inhibits at least two caspase family members, caspase 3 and caspase 7 (Deveraux *et al*, 1997).

A new group a viral inhibitors include the vFLIPs (Hu *et al*, 1997; Thome *et al*, 1997; Bertin *et al*, 1997). These viral inhibitors are members of the death domain family and may represent a new class of viral proteins, able to inhibit Fas and TNFR induced apoptosis by blocking FADD and TRADD killing but unable to inhibit caspase 8 induced apoptosis. This suggests an inhibitory function upstream of the caspases and the presence of the death domains within the members of this viral protein family suggests that these inhibitors might antagonise the FADD-caspase interaction and thereby attenuate Fas- and TNFR-induced apoptosis.

The first mammalian inhibitor designated FLIP is currently investigated by a number of different laboratories, hence the variety of names for the same protein (Hu *et al*, 1997; Srinivasa *et al*, 1997; Shu *et al*, 1997; Irmler *et al*, 1997B; Goltsev *et al*, 1997) (see also chapter 1.5.3.1. and 1.5.3.2.). Its discovery firmly establishes the roles caspases 8 and 10 in the Fas/TNF pathway and might represent the first of a number of specific naturally occurring mammalian caspase inhibitors.

1.6.3.2. Synthetic inhibitors

Synthetic inhibitors have not only proved to be valuable tools in the determination of the catalytic mechanism and the substrate specificities of the caspases but have also given evidence for the direct involvement of caspases in the execution of apoptosis (reviewed by Thornberry *et al*, 1995; Livingston, 1997). Peptide aldehydes, nitriles and ketones are potent reversible inhibitors of the caspase family, while compounds that form thiomethylketone adducts with the active site cysteine are potent irreversible inhibitors (Thornberry *et al*, 1992; Thornberry *et al*, 1994; Thornberry *et al*, 1995). A tetrapeptide corresponding to the substrate residues P4-P1 is sufficient for inhibition for both caspase 1 and caspase 3 and as a consequence has formed the basis for inhibitor design. Peptide inhibitors have since been devised as

reagents to block and thereby implicate the presence of certain caspases in the apoptotic pathway. The tetrapeptide aldehyde Ac-YVAD-CHO was designed to mimic the YVHD caspase 1 recognition sequence within proIL-1β and has proved to be a potent inhibitor of caspase 1. Coupled to a DEAE column, YVAD has been used to purify active ICE to homogeneity from cytosolic extracts, demonstrating the avidity and specificity of the peptide-enzyme binding (Thornberry *et al*, 1992). The tetrapeptide aldehyde Ac-DEVD-CHO was designed to mimic the caspase recognition site within poly(ADP-ribose)polymerase (PARP), a well-characterised caspase substrate, and a biotinylated form of this tetrapeptide has been used as an affinity ligand to purify caspase 3 from cytosolic extracts (Nicholson *et al*, 1995). Subsequent kinetic analysis of a number of other caspase family members, some of which are very similar in sequence to caspase 1 or caspase 3, have shown, however, that neither inhibitor is exclusively effective against those two caspases; both also inhibit other caspase family members, albeit with different potency (Margolin *et al*, 1997). Incubation of a variety of mammalian cells, induced to undergo apoptosis with different stimuli, with both caspase inhibitors not only prevents the activation of multiple caspases, but also prevents the cleavage of substrates and blocks all morphological changes characteristic of apoptosis (McCarthy *et al*, 1997) (recent publications include Kluck *et al*, 1997A; MacFarlane *et al*, 1997; Mariani *et al*, 1997; Wolf *et al*, 1997; Hara *et al*, 1997; Polverino *et al*, 1997; Armstrong *et al*, 1996; Slee *et al*, 1996). Studies with both inhibitors have thus firmly confirmed the status of caspases as effectors of the apoptotic pathway but have left the question of the role of specific caspases mostly unresolved.

Development of the biotinylated peptide YV(bio)KD-aomk, which is based on the caspase 1 cleavage site in pro-IL-1β and only binds to the larger subunit of active caspase 1 and other active caspases, including caspase 3 and caspase 6, allows direct affinity labelling of active caspases (Thornberry *et al*, 1994). YV(bio)KD-aomk applied to cell free extracts allowed visualisation of at least five distinctive polypeptides which correspond to active caspases (Takahashi *et al*, 1996A; Takahashi *et al*, 1996B). Another affinity labelling reagent Z-EK(bio)D-aomk designed to mimic the EVD motif (Thornberry *et al*, 1994) covalently modifies the larger subunit of

caspase 1, 2, 3, 4, 5 and 6. On unidimensional SDS-PAGE the affinity label detects four discrete bands in HL60 cells undergoing apoptosis in response to etoposide treatment, with each band appearing at a characteristic time after the addition of the apoptotic stimulus. Two-dimensional SDS-PAGE revealed that as many as eight different subunits are involved in apoptosis of these cells. Multiple species of caspase 3 and caspase 6 have been identified as the major active caspases in apoptotic cells (Martins *et al*, 1997B; Faleiro *et al*, 1997) These inhibitor studies thus firmly suggest the involvement of multiple caspases in apoptosis. More specific new peptide inhibitors identified in recent specificity studies of caspases, which utilise a positional scanning synthetic combinatorial library, may help to identify the majority of unknown distinctive caspase subunits.

		P4	P3	P2	P1
WEHD	Caspase 1	W	E	H	D
	Caspase 4	W or L	E	H	D
	Caspase 5	W or L	E	H	D
DEXD	*C. elegans* CED3	D	E	T	D
	Caspase 3	D	E	V	D
	Caspase 7	D	E	V	D
	Caspase 2	D	E	H	D
(VL)EXD	Caspase 6	V	E	H	D
	Caspase 8	L	E	T	D
	Caspase 9	L	E	H	D

Figure 1.6. Peptide specificities of individual caspases (adapted from Thornberry *et al*, 1997).

No inhibitor was found which was specific to a single caspase. However, based on their inhibition profile the caspases have been classified into three caspase subclasses which might give better clues to the identities of the enzymes involved in apoptosis and their relationship to each other (Thornberry *et al*, 1997).

1.6.3.3. Knockout mice

Insights into both the importance and the potential redundancy of caspases has been gained from observations in knockout mice. Caspase 1/ICE deficient mice

develop normally, appear healthy and are fertile. The mice are, however, unable to produce mature IL-1β and are highly resistant to the lethal effects of endotoxin by surviving a high dose of lipopolysaccharides (LPS) that kills all wild type mice. Additionally, their macrophages are defective in processing and release of IL-1β but sensitive to ATP-induced apoptosis. Thymocytes from caspase 1 knockout mice have been shown to be sensitive to apoptosis induction by dexamethasone and radiation but are resistant to Fas-induced apoptosis measured by cell viability *in vitro*. The physiological significance of this observation is, however, unclear as analysis of the caspase 1 deficient mice revealed no phenotype resembling the MRL/lpr mice that are defective in Fas-mediated apoptosis (Kuida *et al*, 1995; Li *et al*, 1995; Li *et al*; 1997). Studies of the caspase 2/Nedd2 knockout mice show that these mice develop normally and are healthy. Their B cells, however, show a partial resistance to apoptosis induced by granzyme B and the mice at 4 days after birth possess twice the number of oocytes of wild type litter mates (Bergeron *et al*, unpublished data). Various models can be build around these results, e.g. caspases other than caspase 1 and 2 are essential for physiological cell death, several proteases may function redundantly in apoptosis, or caspase 2 itself plays little role in apoptosis in the majority of tissues, in which case one would predict that mice deficient in one caspase would still be capable of normal apoptosis. The fact that many caspases are coexpressed in many tissues and cell types does suggest some redundancy of the cell death pathway. Evidence against complete redundancy between several caspases comes from observations of caspase 3/CPP32 knockout mice. These are smaller than their littermates, die prematurely at about 1-3 weeks of age and frequently die *in utero* or perinatally. They show profound abnormalities of the central nervous system development, with largely increased number of neurones, a variety of hyperplasias and disorganised cell development. The existence of these excess cells is most likely a result of decrease in the normal rate of apoptosis of neuroepithelial cells (Kuida *et al*, 1996). This demonstrates that mutation of a mammalian *ced3* homologue leads to decreased cell death and a supernumerary cell population during development, suggesting that the basic machinery of apoptosis is evolutionarily conserved. The brain specificity of the caspase 3/CPP32 phenotype also suggests that other caspases may have an important role during apoptosis in

other tissues or cell types. Future knock-outs of various other caspase family members will give a better understanding of the roles of individual caspases and may reveal that the activity some individual caspases is more important in apoptosis than others.

1.6.3.4. Cell free systems

Cell free systems provide another powerful approach for study of the effector mechanisms of apoptosis. They allow dissection of the activation of the effector from the target structure involved in the effector pathway itself. Proteases or their inhibitors can be directly added to the system circumventing the permeability barrier that the plasma membrane presents in intact cells. Cell free systems therefore permit the analysis of the role of individual components of the pathway and provide information about the identity and cellular localisation of the substrates of the effector. In the first description of such a system extracts from chicken hepatoma cells induced in HeLa nuclei all the nuclear morphological changes expected in apoptosis, including chromatin condensation, dissociation of the nuclear membrane and DNA fragmentation (Lazebnik *et al*, 1993). The active principle in these cell free extracts turned out to be a cytoplasmic cysteine protease, named prICE (*p*rotease *r*esembling *ICE*) which provided first evidence for the existence of other caspases apart from caspase 1/ICE. The same extracts also led to the identification of various nuclear caspase substrates (Lazebnik *et al*, 1994; Lazebnik *et al*, 1995A; Lazebnik *et al*, 1995B; Takahashi *et al*, 1996B) (see chapter 1.6.4.). Several other cell free systems have since been described, including extracts from human Jurkat and HL60 cells (Zhivotovsky *et al*, 1997; Muzio *et al*, 1997; Schlegel *et al*, 1996; Martins *et al*, 1997B) and mouse W4 lymphoma cells (Enari *et al*, 1996A) These have proved to be valuable tools for the study of proteolytic activities involved in Fas-mediated apoptosis. Cell free apoptosis inducing extracts from *Xenopus* eggs have revealed the connection between mitochondria, the release of cytochrome c and the activation of caspases (Newmeyer at al, 1994; Kluck *et al*, 1997A; Kluck *et al*, 1997B). Cell free apoptotic extracts of HL60 cells have been used in conjunction with Z-EK(bioD)-aomk, a peptide affinity labelling reagent for caspases which covalently binds to a

variety of caspases (caspase 1, 2, 3, 4 and 6), to delineate the spectrum of caspases activated in HL60 (Martins *et al*, 1997B).

1.6.4. Cellular substrates for caspases

Study of the proteolytically cleaved proteins that appear during apoptosis has helped define the biology of the effector pathway and has provided intriguing if still not definite information of the plurality of the apoptotic caspases themselves. If the caspases are indeed in the final common path for apoptotic effector events then their substrates should include cell components that are responsible for the structural changes observed in apoptotic cell death. Candidate substrates known to undergo proteolytic cleavage in apoptosis include PARP (Kaufmann *et al*, 1993; Lazebnik *et al*, 1994, Schlegel *et al*; 1996), nuclear lamins (Lazebnik *et al*, 1995; Zhitovsky *et al*, 1997), fodrin (Martin *et al*, 1995), U1 small ribonuclear protein particle (Casciola-Rosen *et al*, 1994B), growth arrest specific protein 2 (Gas 2) (Brancolini *et al*, 1995), actin (Mashima *et al*, 1995A; Kayalar *et al*, 1996), sterol regulatory element-binding protein (SREBP-1 and SREBP-2) (Wang *et al*, 1995; Wang *et al*, 1996; Pai *et al*, 1996), GDP dissociation inhibitor (D4-GDI) (Na *et al*, 1996), Retinoblastoma protein (RB protein) (An *et al*, 1996); the catalytic subunit of the DNA dependant kinase (Casciola-Rosen *et al*, 1995; Song *et al*, 1996; Han *et al*, 1996), protein kinase Cδ (Emoto *et al*, 1995), PITSLRE kinases (Beyaert *et al*, 1997), histone H1, topoisomerase I and II (Kaufmann *et al*, 1989; Voelkel-Johnson *et al*, 1995), huntingtin (Goldberg *et al*, 1996), the cell membrane associated adenoma polyposis coli protein (APC) (Browne *et al*, 1994, Browne *et al*, 1998), DNA replication complex C (DSEB/RF-C140) (Ubeda *et al*, 1997), keratin 18 (Caulin *et al*, 1997), Alzheimer-associated presenilins 1 and 2 (Kim *et al*, 1997), DNA fragmentation factor (DFF) (Liu *et al*, 1997) and the related chaperone ICAD (caspase-activated deoxyribonuclease inhibitor) that masks and inactivates a nuclear-seeking nuclease responsible for chromatin cleavage (Sakahira *et al*, 1998). In addition, at least eight proteins that are cleaved early in apoptosis remain unidentified, including proteins of 380, 320, 220, 190, 170, 130 and 80 kDa (Rosen *et al*, 1997). Although all of these have been shown to be specifically cleaved early during apoptosis, with inhibition characteristics typical for caspase family members, not all of these substrates have

been shown to be directly cleaved by caspases *in vitro*. Caspase substrates include PARP (Tewari *et al*, 1995C; Nicholson *et al*, 1995; Gu *et al*, 1995B), nuclear lamins (Lazebnik *et al*, 1995; Takahashi *et al*, 1996B; Orth *et al*, 1996A), the catalytic subunit of the DNA-dependant protein kinase (DNA-PKcs) (Casciola-Rosen *et al*, 1996), U1-ribonucleoprotein (Casciola-Rosen *et al*, 1996), PITSLRE kinases (Beyaert *et al*, 1997), actin (Mashima *et al*, 1997), huntingtin (Goldberg *et al*, 1996), presenilins (Kim *et al*, 1997), DSEB/RF-C140 (Ubeda *et al*, 1997) and APC (Webb *et al*, in publication).

Caspase	*Cleaved substrate*
Caspase 1	pro-IL-1β, PITSLRE kinases, PARP (weakly), U1-70snRNP, pro-caspase 1, procaspase 3, procaspase 4
Caspase 2	PARP (weakly)
Caspase 3	PARP, actin, PITSLRE kinases, huntingtin, preselinins, APC, U1-70 snRPN, DNA/RF-C140, DNA-PK_{cs} p460, procaspase 6, procaspase 7, ICAD
Caspase 4	PARP (weakly), U1-70 snRPN, procaspase 1(weakly), procaspase 3
Caspase 5	?
Caspase 6	PARP, lamin A, B1/B2, C, procaspase 3
Caspase 7	PARP, procaspase 1, procaspase 7, U1-70snRNP
Caspase 8	PARP(weakly), procaspase 2, 3, 4, 6, 7 and 9
Caspase 9	PARP
Caspase 10	PARP, U1-70snRNP, procaspase 3, procaspase 7, procaspase 10

Figure 1.7. Human caspases and their substrates

Since the expression of different caspases in different cells and tissues may vary and since recombinant protein levels *in vitro* might not necessarily represent physiological caspase expression levels *in vivo*, the ascription of a particular substrate in apoptotic cells to a particular caspase *in vitro* has to be seen with some caution and depends heavily on comparison of catalytic cleavage efficiencies. Cleavage of PARP by caspase family members is the best-characterised substrate cleavage event during apoptosis. PARP is a nuclear chromatin associated protein with a molecular weight of 116kDa that catalyses covalent attachment of ADP-ribose units from its substrate

NAD^+ to numerous nuclear proteins including itself. PARP is inactivated during apoptosis by cleavage into two fragments of about 25kDa and 85kDa, which results in the isolation of the DNA binding domain from the catalytic domain. PARP cleavage occurs at a DEVDG cleavage site (Lazebnik *et al*, 1994), which is strikingly similar to DEVDN cleavage site of caspase 3 within DNA-PKcs (Casciola-Rosen *et al*, 1996) and the DGPDG cleavage site of caspase 3 within the U1-ribonuclear protein (Casciola-Rosen *et al*, 1996) and strongly supports that caspase 3 is responsible for PARP cleavage. The exact physiological function of PARP and its cleavage in apoptosis, however, is unclear as recent findings from PARP knock-out mice by three different laboratories suggest that neither activation nor cleavage of PARP has a causal role in apoptosis (Leist *et al*, 1997; Wang *et al*, 1997; Eliasson *et al*, 1997). PARP may play an important role in the maintenance of genomic integrity and activation of PARP is a key mediator of excitotoxic and ischemic neuronal and non-neuronal cell death rendering PARP knock-out mice resistant to cerebral ischemia.

Cleavage of structural proteins such as fodrin, actin and keratin as well as the nuclear lamins by caspases could all contribute to the altered cell shape and rearrangements during apoptosis. Apoptosis is, however, also accompanied by the internucleosomal degradation of chromosomal DNA (see chapter 1.2.). Recent findings by S. Nagata's group in mice show that chromatin is cleaved by a nuclease that is specifically activated by caspase 3 during apoptosis, and has thus been named CAD (caspase-activated DNase) (Enari *et al*, 1998; Sakahira *et al*, 1998). CAD is present in the cytoplasm in its inactive form bound to and stabilised by its cytoplasmic inhibitor ICAD (inhibitor of CAD). Upon caspase 3 activation ,triggered by different apoptotic stimuli, ICAD is cleaved by caspase 3, releases CAD, which enters the nucleus and cleaves DNA. The caspases thus may not only directly cleave critical elements of the cell in the ordered process of apotosis, but also trigger, through cleavage of specific inhibitors, downstream events like DNA degradation. While some events appear to be triggered in parallel (e.g. U1-70 and PARP cleavage) others may be in series, possibly because of a cascade-like central organisation of the caspases. The caspases, thus have the capacity to contribute to multiple near-synchronous events in the nucleus and

in the cytoplasm that underlie the long-known characteristic structural events of apoptosis. How this activity of the caspases is regulated is poorly understood.

1.6.5. Regulation of the caspases

As described above, application of both cell-permeable synthetic peptide and natural, macromolecular inhibitors, generation of knockout mice as well as the identification of several potential downstream targets have established the role of caspases as the effector molecules of apoptosis. How this protease activity is activated and regulated and where the caspases act in the pathway in relation to other important regulatory molecules is beginning to be resolved. Multiple levels of control probably exist for the regulation of caspase activity within the cell. In theory control could be achieved at the mRNA level through transcriptional regulation and alternative splicing and at the protein level through posttranslational modification, proteolytic cleavage and interaction with other components of the cell death pathway.

Northern blot analysis demonstrates that mRNA expression of all caspases can be detected in most human tissues. Caspase 1 mRNA has been shown to be induced by loss of adhesion to extracellular matrix and by transcription factor IRF-1 (Boudreau *et al*, 1995). *De novo* synthesis of procaspase is, however, not required for the existence of caspase activity during apoptosis as treatment of cells with cycloheximide or puromycin does not inhibit cellular caspase activity (Weil *et al*, 1996; Martins *et al*, 1997B).

Northern blotting further reveals the presence of splice variants of some of the family members. Four isoforms of Caspase 1/ICE have been cloned β, χ, δ and ε (Alnemri *et al*, 1995). Isoforms of other caspases, including caspase 2/ICH-1 (termed ICH-1_L and ICH-1_S), caspase 3/CPP32 (CPP32α and β), caspase 4/Mih4 (α, β, χ, δ), caspase 6/Mch2 (Mch2α and Mch2β), caspase 7/Mch3 (α and β) and caspase 10/Mch4/FLICE2 (α and β) have also been identified (see chapter 1.6.1. for original references). These isoforms arise by the usage of alternative splice donor/acceptor sites and result in a full-length isoform of the enzyme and truncated forms. Some of these caspase isoforms have opposing functions acting as inducers or inhibitors, including caspase 1, 2, 6 and 7, whereas isoforms of caspase 3, 4 and 10 are all inducers of apoptosis. The presence of some caspase isoforms in apoptotic cells,

including caspase 3 and 6, has been shown by the use of biotinylated peptide affinity labels (see chapter 1.6.3.2.). The biological role of certain isoforms, particularly caspase 2 (ICH-1_S), is, however, controversial as transfection of FDC-p1 cells with *NEDD2*$_S$, the mouse equivalent of *ICH-1*$_S$, has no effect on cell survival and therefore does not suggest a major role in the regulation of apoptosis, at least in that cell type (Kumar, 1995). Potentially enzymatically inactive, alternatively spliced isoforms could regulate the enzyme activity by acting as dominant negative inhibitors of the enzyme, perhaps through interference with the heterodimerisation of the active full length form of the enzyme. Proof of this principle is provided by a synthetic active site mutant of caspase 10 which has a dominant negative effect and effectively inhibits apoptosis (Vincenz *et al*, 1997). The mammalian caspase inhibitor FLIP also exists in several isoforms (see also chapter 1.5.3.1.). Itself catalytically inactive, it could be processed to inactive subunits which then, on the basis of high sequence identity, heterodimerise with either of the caspases thus serving as a dominant inhibitor (Hu *et al*, 1997). The exact nature of the inhibitory mechanism of alternatively spliced caspase and caspase inhibitor isoforms still needs to be investigated. Alternatively to acting as dominant negative inhibitors, isoforms with potentially different structure could interact with activators of the caspases and prevent activation of the full length caspase proenzymes.

So far no information is available on posttranslational modification, including glycosylation or phosphorylation of the caspases. However, the presence of multiple caspase subunits in apoptosis, detected with biotinylated affinity labels on two-dimensional SDS-PAGE could indicate differences in posttranslational modifications. Ubiquitination has been shown to regulate the turnover of several proteins that might be involved in apoptosis, including cyclins, c-myc and p53 (Ciechanover, 1994). Although the role of the ubiquitin/proteasome pathway in apoptosis is unclear, recent evidence links proteasome inhibition to caspase 3 activation (Drexler, 1997).

Post-translational activation by proteolytic cleavage is a general feature of the caspase family as described earlier (see chapter 1.6.2.). Most members of the caspase family exhibit autocatalytic cleavage and activation when overexpressed in bacteria as proenzymes. The extent to which this autoactivation occurs in authentic apoptosis is

still uncertain, but the process could be critical for the initiation of activity of "apical caspases", activated by their local high concentration on recruitment to the cytosolic face of receptors such as Fas and their associated binding proteins.

A variety of studies have now confirmed that caspases of differing specificities are required for the complete execution of apoptosis as different inhibitors with a variety of specificities inhibit the apoptotic process and the cleavage of certain substrates at various points during the apoptotic process (Fearnhead *et al*, 1995; Zhitovsky *et al*, 1995; Takahashi *et al*, 1996A; Takahashi *et al*, 1996B; Zhou *et al*, 1997; Longthorne *et al*, 1997). Several lines of evidence suggest that this activation occurs as a protease cascade. Thus various caspases have been shown to have protease activity against other procaspases (listed in Figure 1.7.). Caspase 4/TX when overexpressed in COS cells has protease activity against procaspase 1 (Faucheu *et al*, 1995). Caspase 7/Mch3α has a potential cleavage site (amino acids 20-23, DSVD) which is very similar to the caspase 3 tetrapeptide substrate DEVD, making caspase 7 a potential substrate for caspase 3 (Alnemri *et al*, 1995). Purified caspase 3 can cleave and activate procaspase 2 (Nedd2), 6 and 7 and can in turn be cleaved by purified caspase 1, caspase 6 and caspase 8 (FLICE) (Fernandes-Alnemri *et al*, 1995; Tewari *et al*, 1995D; Harvey *et al*, 1996). Also fine data on time course studies shows that caspase 8 (FLICE) is capable of processing the majority of procaspases in a sequential order. It can directly or in the presence of cell free extracts activate caspase 2, 3, 4, 6, 7 and 9 (Muzio *et al*, 1997). Thus activation of one caspase could lead to an amplifiable protease cascade with caspase 8 and 10 being potentially the most apical members (see below). The prodomain of individual caspases might play an important role in caspase activation. Generally proapoptotic caspases can be divided into two groups: those with a large prodomain such as caspase 2, 8, 9 and 10 and those with small prodomains such as caspase 3, 6 and 7. Caspases with large prodomains are probably the most upstream caspases (Srinivasula *et al*, 1996A, Srinivasula *et al*, 1996B; Ahmad *et al*, 1997A) recruited directly by the Fas/TNF pathway. In the case of caspase 2, caspase 8 and caspase 10 the prodomain containing death effector domain motifs can interact with specific regulatory molecules and allow differential activation by different death inducing signals (see chapter 1.5.3.).

Caspases can also be activated by other proteases. Granzyme B gave the first indication that other non-caspases interact and are part of this protease cascade. Thus, the serine protease granzyme B from the granules of cytotoxic T lymphocytes activates caspase 3 and cleaves caspase 1, caspase 2 (ICH-1/Nedd2) and caspase 8. Other caspases including caspase 6, 7 and 10 might also be activated by granzyme B (Darmon *et al*, 1995; Quan *et al*, 1996; Harvey *et al*, 1996; Muzio *et al*, 1996; Vincenz *et al*, 1997; Alnemri, 1997).

Further control of enzyme activity might be achieved through heterooligomerisation. Genetic complementation experiments have shown that several members of the caspase family can heterodimerise with one another and form functional intermolecular heterocomplexes that can also compensate for mutational inactivation of individual subunits (Fernandes-Alnemri *et al*, 1995B; Faucheu *et al*, 1995; Gu *et al*, 1995). The two closely related caspases 3 and 7 can form functional intermolecular heterocomplexes and caspase 1 and 4 form heterooligomers.

Dramatic advances have been made in the last two years regarding the order of the cell death pathway components and the upstream regulation of caspase activity (see also chapter 1.4.1.). Biochemical studies investigating caspase activation, PARP cleavage and apoptotic morphology have placed Bcl-2, Bcl-xl and the adenovirus E1B 19kDa protein, a viral homologue of Bcl-2, clearly upstream of the caspases (Chinnaiyan *et al*, 1996A; Boulakai *et al*, 1996; Ibrado *et al*, 1996; Shimuzu *et al*, 1996). Clues to how Bcl-2 family members regulate the activation of caspases comes from studies on the role of mitochondria in apoptosis and links caspase activation to cytochrome c release (Newmeyer *et al*, 1994; Kluck *et al*, 1997A; Kluck *et al*, 1997B). Cytochrome c has been shown to be released from the mitochondria of staurosporine induced HL-60 cells and Jurkat cells induced by Fas ligation and leads to the induction of apoptosis characterised by lamin B_1 and fodrin cleavage and activation of caspases (Yang *et al*, 1997; Adachi *et al*, 1997).

1.6.6. Caspases: Some remaining questions

Major advances in apoptosis research in the last three years have firmly established the role of caspases as the effector molecules of apoptosis. Unresolved is whether all the caspases identified take part in the apoptotic pathway and whether the

caspases may have other physiological functions in addition to effector molecules in apoptosis. Whether caspase participation is stimulus or tissue specific is also only partially resolved. Nor is it clear whether caspases have preferred intracellular localisations (before and after activation), and if so whether this is lineage dependent. The experimental work in this thesis was a step towards answering some of these questions with reference to a caspase discovered early, but as yet with little firmly established physiological function, caspase 2.

2.0 AIMS

My ultimate goal was to define the role of caspase 2 in apoptosis. Specifically, I wished to analyse its subcellular localisation, subunit configuration, expression and tissue distribution in both normal and diseased tissue and its activation during apoptosis. To address this, tools were needed for immunohistochemical, immunocytochemical and Western blotting analysis. At the onset of this work no commercially suitable antibodies were available or even reported in the literature. My aims in this study therefore were a) to generate three different polyclonal antibodies, specific to the zymogen and the subunits of the active enzyme, using as immunogens bacterially expressed recombinant polypeptides, specific to functional regions of caspase 2; b) to establish the specificity of those antibodies and c) to use those antibodies to delineate caspase 2 in cells and tissues and to investigate the relationship of caspase 2 to the initiation of apoptosis. To examine this, apoptotic cell culture systems of two mammalian cell lines were developed and caspase 2 levels were studied in response to a variety of different apoptotic stimuli, including a nuclear (topoisomerase II inhibition), a cytoplasmic (protein kinase inhibition) and a cell surface (Fas receptor coupling) stimulus.

3.0 RESULTS

3.1 CASPASE 2 RECOMBINANT POLYPEPTIDES AS RABBIT ANTIGENS

3.1.1. Design of procaspase, large and small subunit specific caspase 2 antigens

In order to raise antibodies to caspase 2 which do not cross-react with other caspase family members, amino acid regions within caspase 2 with low homology to other known caspases had to be identified. These less conserved and caspase 2 specific amino acid regions would then be subsequently expressed in bacteria and would be used as antigens for immunisation to obtain specific rabbit anti-caspase 2 polyclonal antibodies. A protein sequence alignment of the identified caspases at that time was carried out. This included human caspase 1, caspase 2 and caspase 3 as well as the *C. elegans* CED3 protein (Figure 3.1.).

Alignment of these four caspase sequences indicated that all four caspases contain a number of highly conserved key amino acids necessary for enzyme activity. Regions of highest identity between the four caspases were found in the small and large subunit, particularly around the active site cysteine and histidine with the pentapeptide QACRG conserved between the individual caspases. A lower region of similarity was apparent in the prodomains of the caspases.

Parts of the caspase 2 prodomain sequence thus seemed particularly promising caspase 2 specific epitopes, whereas the active site pentapeptide and adjacent amino acids were found to be so highly conserved that the risk of cross-reactivity between individual caspases would be high. Lesser conserved amino acid stretches at the COOH-terminal and in the center of caspase 2 seemed also suitable as caspase 2 antigen.

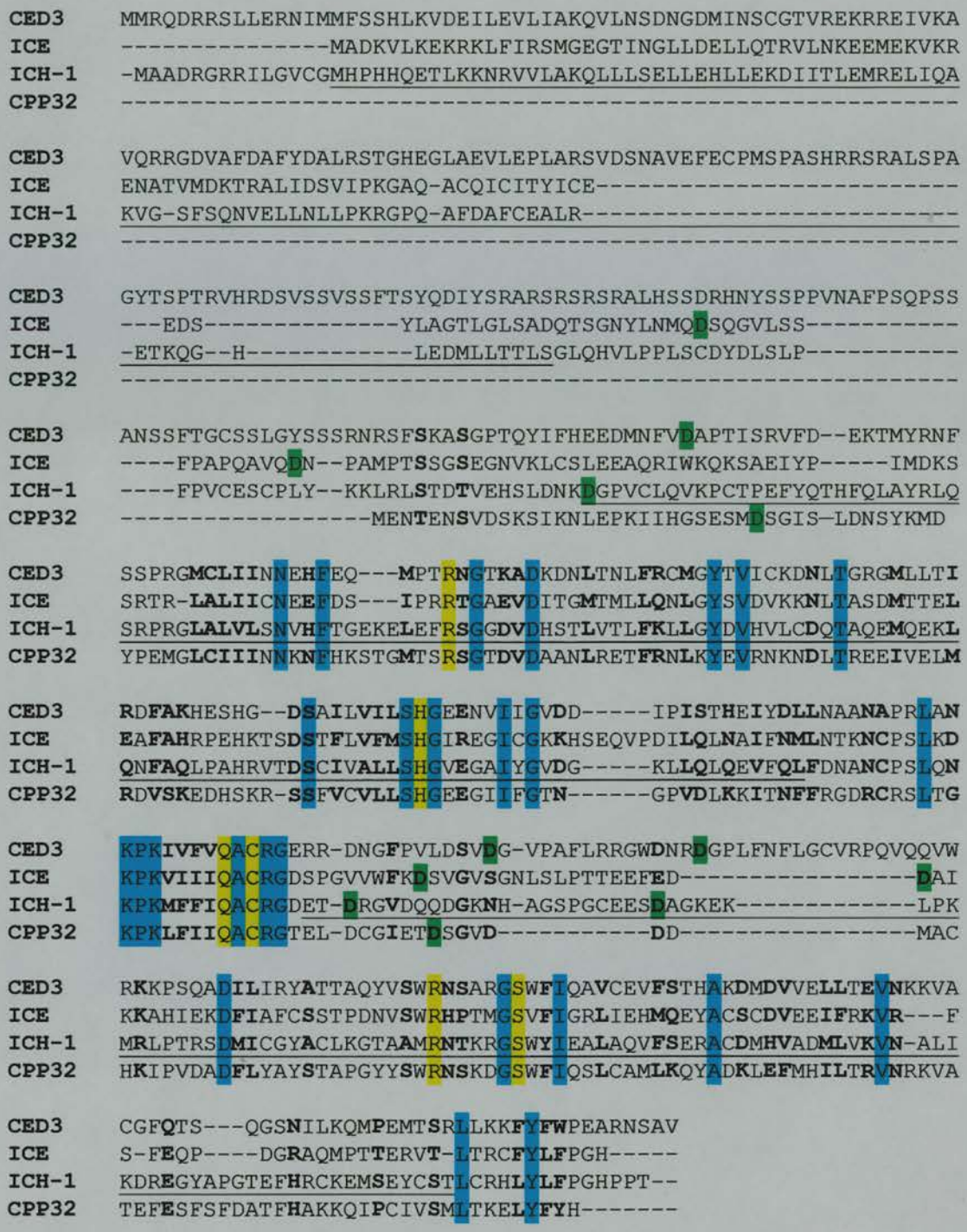


Figure 3.1. Sequence alignment of multiple caspase family members. Sequence alignment of caspases 1 (ICE), 2 (ICH-1l/s), 3 (CPP32) and CED3. Identical amino acid residues are marked in blue and yellow within the sequence. Amino acid residues required for enzyme catalysis are marked in yellow. Amino acid residues at positions, which tend to be of conservative character, are printed in bold. Aspartic acid proteolytic cleavage sites giving rise to the active enzyme (undetermined for caspase 2 and CED3 at the time of the original analysis) are highlighted in green for each individual caspase sequence. Caspase 2 polypeptide antigen sequences are shown as underlined amino acid residues.

One further important factor to be considered in the choice of caspase 2 antigen is the position of the post-translational processing sites. Alignment of the caspase 2 sequence with that of caspase 1 permitted prediction of the processing sites of caspase 2 and hence identification of the prodomain, the large p18 and the small p12 subunit (Figure 3.2.). Peptide sequences were selected to lie within these regions. Subsequently published microsequencing data on caspase 2 confirmed the validity of these choices, with the exception of an additional 20 amino acids in the NH_2-terminal region of fragment 2, which lie within the linker region that is cleaved off upon enzyme activation. The remaining 86 amino acids of fragment 2 do, however, all lie within the p12 subunit.

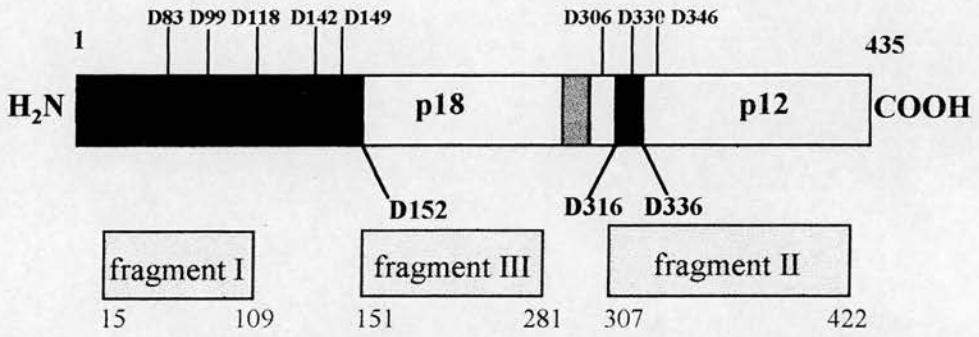


Figure 3.2. Subunit specific caspase 2 antigens. A schematic representation of caspase 2/ICH-1_L. The prodomain and linker regions are indicated as solid black boxes. The size and the position of the protease subunits is depicted within the precursor protein, the predicted active site pentapeptide is indicated by a solid dark grey box. Vertical lines above the diagram indicate the potential precursor aspartic acid cleavage sites to form the active enzyme subunits and are numbered according to their position in the protease precursor. The actual cleavage sites are indicated in bold underneath the diagram. The chosen three recombinant caspase 2 polypeptide sequences (fragment I-III) and their relative positions within procaspase 2 are indicated as light grey boxes.

Three polypeptide sequences within caspase 2 were thus chosen as antigens (see also Figure 3.1. for underlined caspase 2 amino acid sequence). The predicted molecular weight of the three caspase 2 fragments is 11.9kDa for the NH_2-terminal prodomain fragment (polypeptide/fragment 1), 13.7kDa for the COOH-terminal small subunit fragment (polypeptide/fragment 2) and 15.4kDa for the large subunit fragment

(polypeptide/fragment 3). The resulting anti-fragment 2 and anti-fragment 3 antibodies will recognise either of the active enzyme subunits and the proenzyme containing the uncut subunits, whereas the anti-fragment 1 antibody will only bind to the proenzyme. These antibodies will thus allow the study of caspase 2 activation in apoptosis, analysing the appearance of both subunits, and will also allow comparative immunohisto- and immunocytochemical studies of protein levels of caspase 2 proenzyme versus active enzyme.

3.1.2. Construction of expression vectors for caspase 2 fragments in bacteria

In order to amplify the *caspase 2* coding region, total mRNA was isolated from HeLa cells. The polynucleotide sequences corresponding to the three caspase 2 polypeptide fragments and the whole sequence were amplified with specific primers by RT-PCR and analysed by agarose gel electrophoresis (Figure 3.3.).

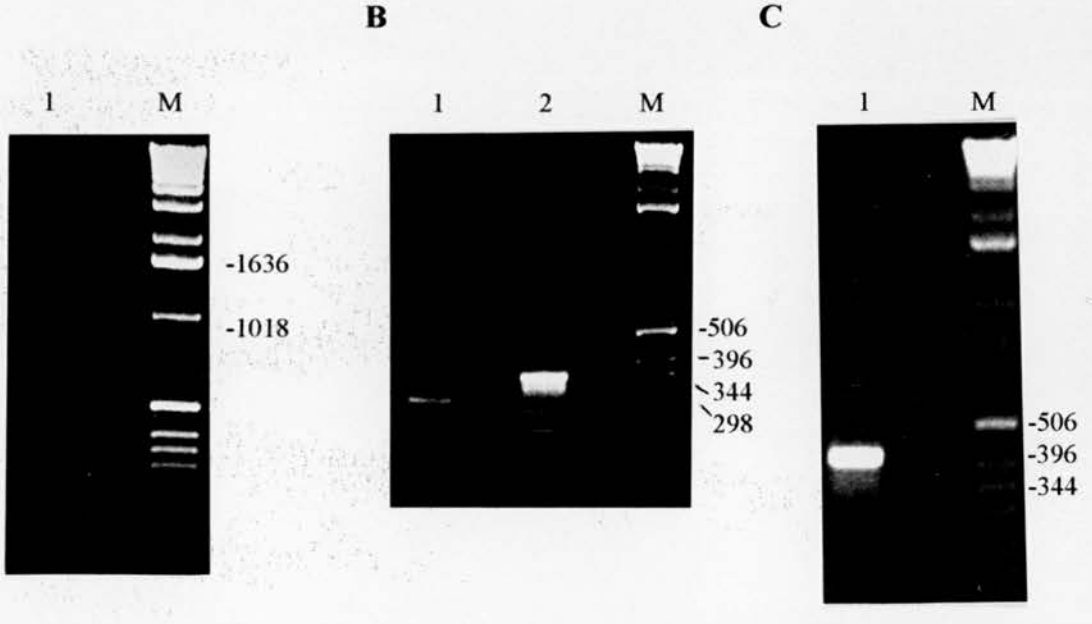


Figure 3.3. RT-PCR amplification of three *caspase 2* (*ICH-1L*) oligonucleotide fragments. M: marker in base pairs; **A)** lane 1: whole *caspase 2* cDNA; **B)** *lane 1*: fragment 1; *lane 2*: fragment 2; **C)** *lane 1*: fragment 3.

The predicted open reading frame of *caspase 2* cDNA consists of 1308 bp and encodes the 435 amino acid caspase 2 (ICH-1_L) protein product. The first caspase 2 fragment (prodomain fragment) is encoded by 282 bp, the second fragment (small

subunit fragment) is encoded by 348 bp and the third fragment (large subunit fragment) is encoded by 408 bp. All three fragments and the entire coding region, with the correct size, were amplified from the cDNA (Figure 3.3.). The position of the amplified *caspase 2* fragments 1-3 within the *caspase 2* cDNA sequence is indicated in Figure 3.4.

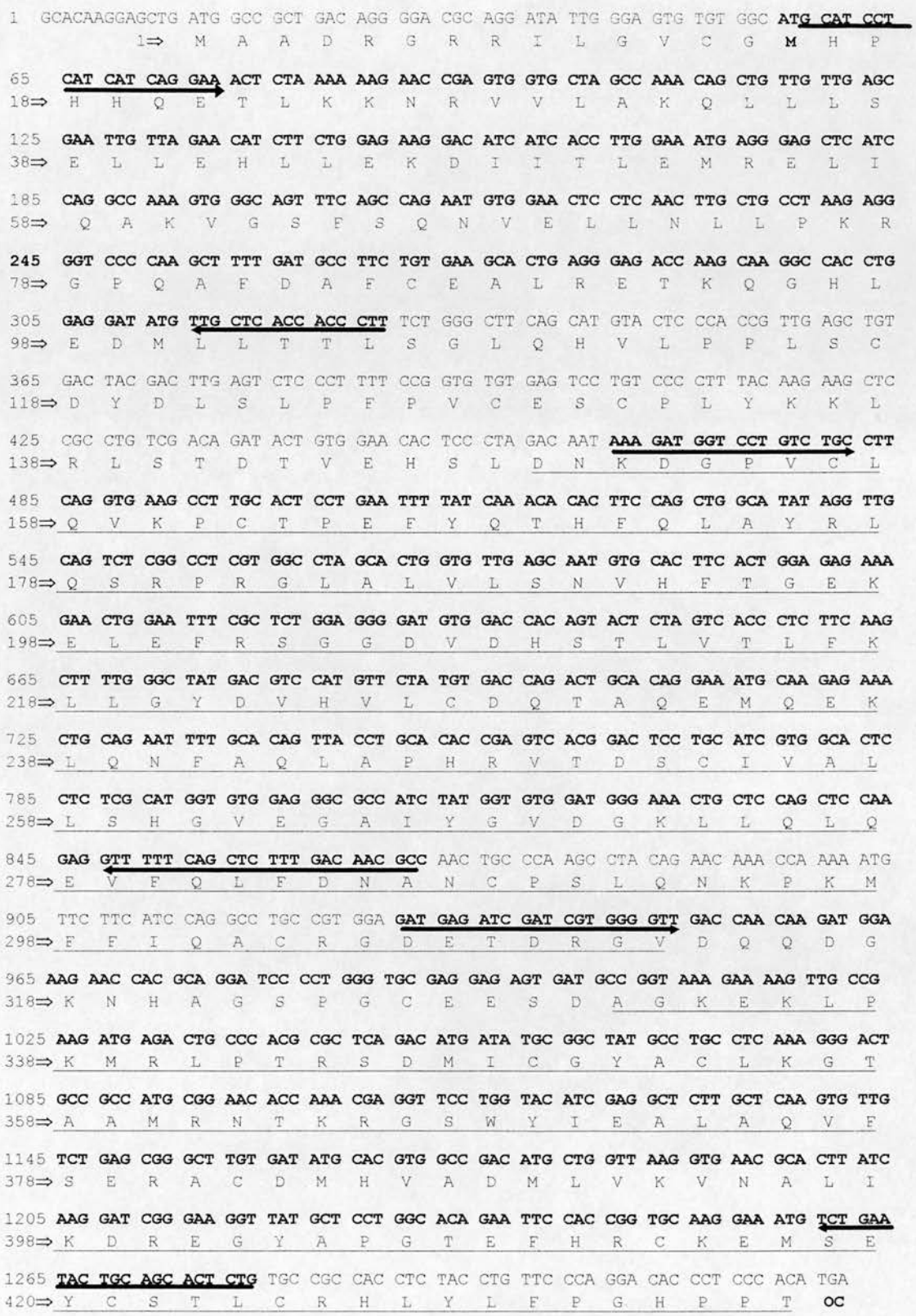


Figure 3.4. Nucleotide and deduced amino acid sequence of *caspase 2 (ICH-1L)* Nucleotides are numbered 5' to 3'. The termination codon is designated OC. The primers are denoted by bold arrows, PCR amplified polynucleotide sequences are in bold print. The active enzyme subunit composition is underlined.

In order to construct caspase 2 expression vectors, PCR-amplified caspase 2-polynucleotide fragments 1-3 were digested with XhoI and NcoI and were ligated with linearised pET23d bacterial expression vector. The ligated vectors were transformed into competent cells and analysed by a single restriction digest with the restriction endonuclease PvuI, which cuts the pET23d vector at a single site resulting in vector linearisation or a double digest with the restriction endonucleases DraIII and BglII, which each cut the vector at a single site (see Appendix 2, Figure 1. pET23d vector diagram).

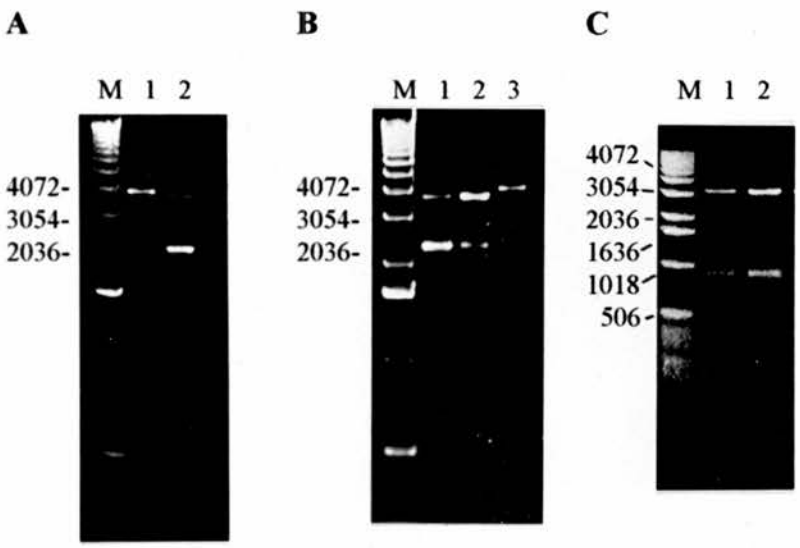


Figure 3.5. Restriction endonuclease analysis of recombinant pET23d vectors 1, 2 and 3. M: kilobase marker in base pairs. **A)** incomplete PvuI digest; *lane 1*: top band (very faint), nicked circular recombinant vector; middle band, linearised recombinant vector pET23d1 (insert: fragment 1) 3865bp; lower band supercoiled recombinant vector. **B)** incomplete PvuI digest; *lane 1-2*: top band, nicked circular vector; middle band, linear pET23d without insert 3583bp; lower band, supercoiled vector; *lane 3*: recombinant vector pET23d2 (insert: fragment 2) 3931bp. **C)** DraIII and BglII digest; *lane 1-2*: linear recombinant vector pET23d3 (insert: fragment 3) 3090bp and 901bp.

The restriction digest gel analysis of clones showed the presence of predicted DNA fragments of the appropriate size corresponding to recombinant vectors pET23d1-3 (Figure 3.5.). The correct cloning of the *caspase 2* polynucleotide fragments 1-3 in the recombinant vectors pET23d1-3 was further confirmed in a restriction

endonuclease digest with NcoI and XhoI, the original cloning sites for all three polynucleotide fragments, resulting in the excision of the cloned *caspase 2* fragments (data not shown).

In order to exclude any mutations and to confirm correct insertion of the fragment into the vector in frame with the six COOH-terminal histidine residues, all three plasmids (pET23d1-3) were subsequently sequenced. Correct in frame insertion of the target DNA into the linearised vector is of critical importance to ensure translation of the COOH-terminal histidine residues which allow the purification of the recombinant protein from bacterial lysates on nickel columns by affinity chromatography (see also chapter 3.1.3.). No mutations within caspase 2 polynucleotide sequences were found and the correct reading frame was confirmed (sequencing data not shown).

3.1.3. Preparation and purification of bacterial lysates expressing caspase 2 polypeptides

To obtain recombinant caspase 2 polypeptide fragments as antigens for the immunisation of rabbits, the three recombinant plasmids were transformed into the *E.coli* bacterial expression strain BL21 (DE3) pLysS. This bacterial expression strain is a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promotor and the gene for T7 RNA polymerase. The only promotor known to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promotor, which is inducible by the nonmetabolisable analog of lactose, IPTG. Addition of IPTG to a growing bacterial culture induces T7 RNA polymerase, which in turn transcribes the target DNA in the pET23d vector. The *E.coli* bacterial expression strain BL21 (DE3) pLysS is thus particularly suitable for potentially toxic target gene expression (see Appendix 2, Figure 2. Control elements of the pET system). Target gene expression in the bacterial culture was monitored by SDS-PAGE. Optimal expression was achieved when the growing culture was induced by the addition of IPTG to the culture medium at an optical density (OD_{600}) around 0.6. Overgrowth of the culture to OD> 0.8 resulted in very low yields of recombinant protein.

Initial column chromatography was carried out with each culture extract prepared under native and denaturing conditions. Attempted purification of prodomain caspase 2 fragment 1 and small subunit fragment 2 under native conditions resulted in very

low amounts of recombinant protein (>0.5mg from a 400ml culture). This suggested that both of the recombinant caspase 2 fragments were expressed mainly in insoluble, aggregated form in the bacterial expression strain and were most likely contained within bacterial exclusion bodies. Caspase 2 fragments 1 and 2 were thus subsequently purified from a membrane fraction under highly denaturing conditions in the presence of 6M urea in the binding buffer. In contrast the large subunit fragment 3 appeared to be mainly expressed as a soluble form and was purified under native conditions from the cytoplasmic fraction without further incubation with 6M urea.

To assess recombinant protein yield from induced bacterial cultures, protein concentrations of column purified recombinant protein and soluble and insoluble whole protein fractions were determined at all stages of the lysate preparations. Individual standard curves were prepared each time and Table 3.1. shows the average protein amounts obtained in five separate experiments.

	Culture pET23d1	**Culture pET23d2**	**Culture pET23d3**
Fraction 1	104.32mg	116.83mg	98.65mg
Fraction 2	7.65mg	6.81mg	15.83mg
Fraction 2A	18.99mg	13.19mg	
Fraction 3	6.09mg	3.06mg	1.47mg

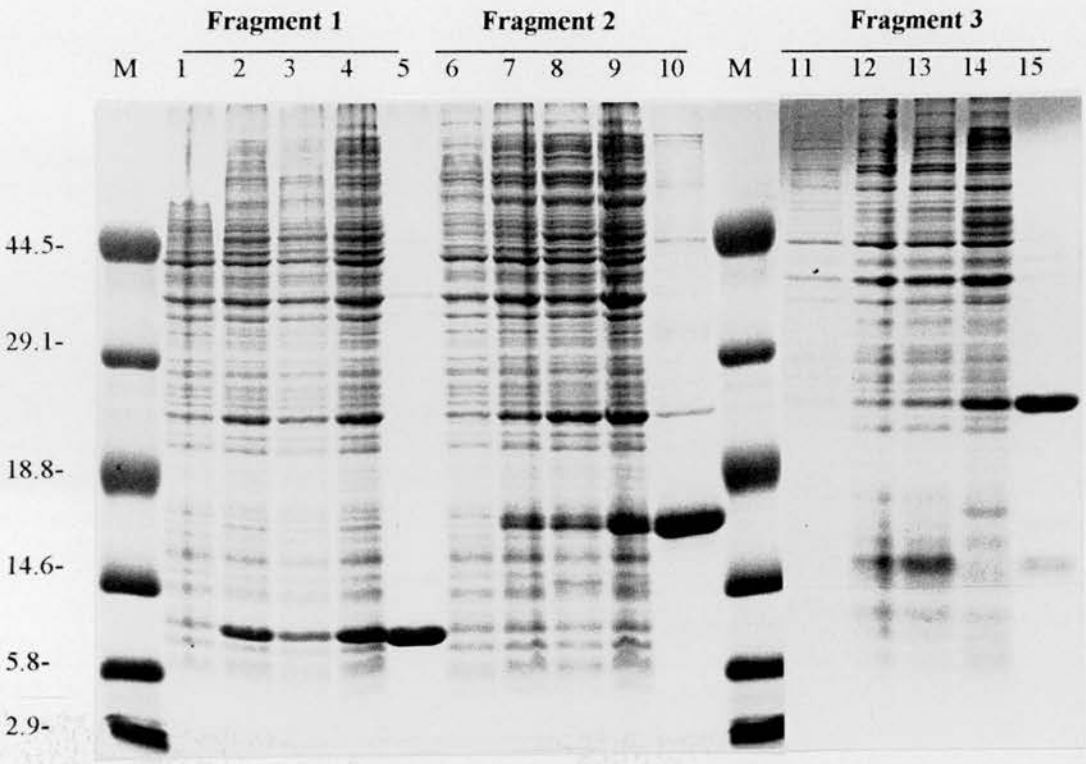
Table 3.1. Protein concentrations of protein fractions obtained from 400ml induced bacterial culture. The OD_{595} values were read from individual standard curves. Protein concentrations represent the average of five independent experiments. Fraction 1 is the whole cell extract after freeze/thaw cycles. Fraction 2 is the soluble protein fraction for culture pET23d3 (discarded for culture pET23d1 and 2) after ultracentrifugation and prior to column chromatography. Fraction 2A is the 6M urea solubilised protein fraction of culture pET23d1 and 2 after ultracentrifugation and prior to column chromatography. Fraction 3 is the column purified eluate fraction, consisting of the purified recombinant protein.

On average substantially less column purified recombinant protein than claimed by the manufacturer was obtained from each induced culture, with particularly low recovery obtained with fragment 3, and the experiment was accordingly scaled up from a recommended 100ml culture volume to 400ml culture. Reasons for the

disappointingly low expression are obscure, but the recombinant caspase 2 fragments could interfere with the integrity of the cell and could be potentially toxic.

In order to monitor target gene expression and purity of the column-eluted caspase 2 fragments samples of total cell protein and column eluted caspase 2 fragments from induced cultures were subjected to protein electrophoresis followed by Coomassie blue staining (Figure 3.6.A). The efficiency of the cell extract preparation for column chromatography by freeze/thaw cycles was assessed by additional analysis of the solubilised final cell pellet (the pellet obtained by ultracentrifugation of lysates prior to column loading) by SDS-PAGE (Figure 3.6.B, data shown for fragment 1 and 2 only).

A


Fragment 1
Fragment 2
Fragment 3
M 1 2 3 4 5 6 7 8 9 10 M 11 12 13 14 15
44.5-
29.1-
18.8-
14.6-
5.8-
2.9-

B

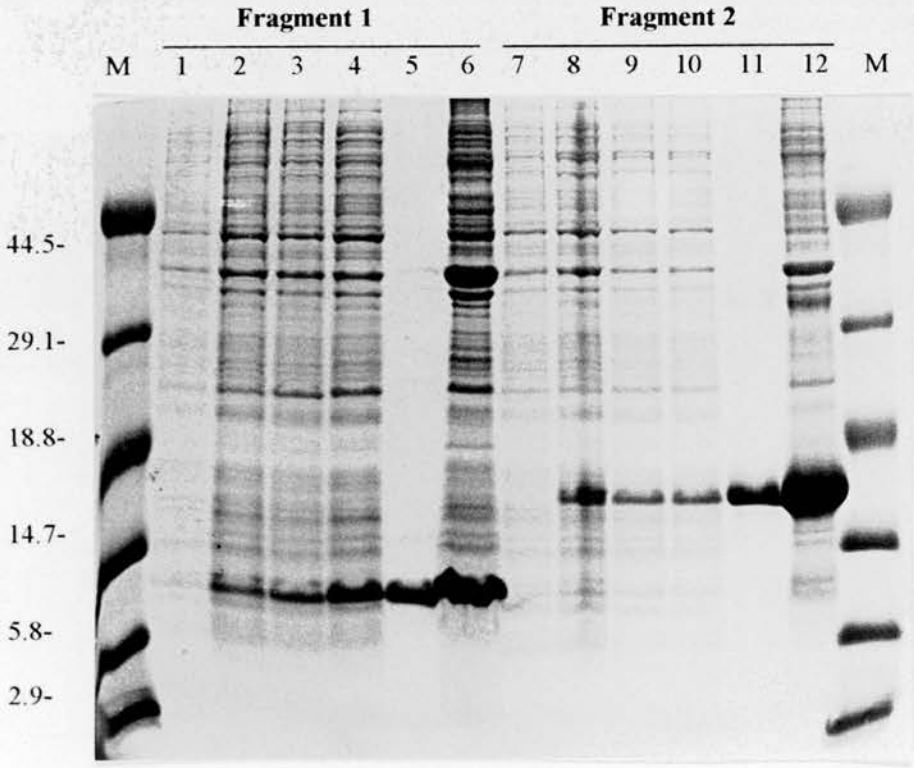

Fragment 1
Fragment 2
M 1 2 3 4 5 6 7 8 9 10 11 12 M
44.5-
29.1-
18.8-
14.7-
5.8-
2.9-

Figure 3.6. SDS-PAGE analysis of recombinant caspase 2 fragment expression. Expression analysis of recombinant caspase 2 fragments 1, 2 and 3 bacterial cultures lysates and purity analysis of purified recombinant protein. Approximately 50μg/lane of samples protein were run on 15% SDS-PAGE large gel; M: molecular weight standards in kDa. **A)** Culture pET23d1 (fragment 1) lanes 1- 5; culture 23pETd2 (fragment 2) lane 6-10 and culture 23pETd3 (fragment 3) lane 11- 15. *Lane 1*: uninduced total cell lysate; *lane 2*: 1h induced total cell lysate; *lane 3:* 2h induced total cell lysate; *lane 4:* 3h induced total cell lysate; *lane 5:* column purified eluate fraction (fragment 1); *lane 6*: uninduced total cell lysate; *lane 7*: 1h induced total cell lysate; *lane 8:* 2h induced total cell lysate; *lane 9:* 3h induced total cell lysate; *lane 10:* column purified eluate fraction (fragment 2); *lane 11*: uninduced total cell lysate; *lane 12*: 1h induced total cell lysate; *lane 13:* 2h induced total cell lysate; *lane 14:* 3h induced total cell lysate; *lane 15:* column purified eluate fraction (fragment 3). **B)** Culture pET23d1 lanes 1-6; *lane 1*: uninduced total cell lysate; *lane 2*: 1h induced total cell lysate; *lane 3:* 2h induced total cell lysate; *lane 4:* 3h induced total cell lysate; *lane 5:* column purified eluate fraction (fragment 1); *lane 6*: solubilised pellet after ultracentrifugation. Culture 23pETd2 lanes 7-12; *lane 7*: uninduced total cell lysate; *lane 8*: 1h induced total cell lysate; *lane 9:* 2h induced total cell lysate; *lane 10:* 3h induced total cell lysate; *lane 11:* column purified eluate fraction (fragment 2); *lane 12*: solubilised pellet after ultracentrifugation.

The recombinant caspase 2 fragments expressed in the bacterial cultures were visible on the Coomassie stained polyacrylamide gel by the appearance of a new protein band in the total cell lysate at 1 hour after the induction with IPTG. The intensity of bands and the relative amounts of the protein of all three bands corresponding to the recombinant caspase 2 fragments increased over the three hour time period following induction with IPTG.

The prodomain fragment 1 with a predicted molecular weight of 11.9kDa appeared as band of a similar apparent molecular weight in the total cell lysate on a 15% SDS-PAGE gel. In the total cell lysates of the induced cultures expressing fragments 2 and 3, however, the apparent molecular weight of the expressed proteins differed from the expected size.

The small subunit fragment 2 had an apparent molecular weight of 16.5kDa instead of the predicted molecular weight of 13.7kDa. The reason for this apparent discrepancy in molecular weight are obscure.

In several cultures transformed with the pET23d3 vector for the expression of fragment 3, a protein of approximate molecular weight of 22kDa appeared to be strongly induced and was assumed to be caspase 2 fragment 3, which has a predicted

molecular weight of 15.4kDa. Later examination showed, however, that a protein band of similar molecular weight (22kDa) also appeared in the cell lysate of the induced cultures transformed with pET23d1 and pET23d2 and in the column purified fraction of recombinant fragment 2 (Figure 3.6.A, lane 10). However, lysates of bacterial cultures transformed with nonrecombinant pET23d as a negative induction control, also showed a prominent protein band of about 22kda, but no change in expression levels was apparent over a three hour time period, following induction with IPTG (data not shown). In contrast with this prominent band, a weakly induced smaller protein is apparent as a band of approximately 15.5kD in the total cell lysate of pET 23d3 transfected bacteria following 1 and 2 hours after the induction with IPTG, corresponding to the predicted molecular weight of caspase 2 fragment 3. This protein also elutes off the Nickel column, even though in much smaller amounts, and, as later confirmed by amino acid sequencing and mass spectrophotometry (see below), is the recombinant caspase 2 fragment 3.

All three recombinant caspase 2 fragment eluate fractions, purified by metal chelating affinity chromatography (see Figure 3.8. A, lanes 5, 10 and `15), contained some higher molecular weight contaminating proteins. The presence of higher molecular weight proteins is most likely an indication of insufficient washing of the columns prior to elution of Nickel bound histidine-tagged proteins, resulting in retention of unbound larger molecular weight proteins. Due to the relative insensitivity with which Coomassie stain binds to and therefore detects less abundant proteins on fixed polyacrylamide gels, however, no definite answer to the purity of the recombinant protein following column chromatography could be obtained by this method alone.

The inefficiency of bacterial cell lysis by freeze/thaw cycles became apparent (Figure 3.8. B, lane 6 and 10) when the final cell pellet, obtained by ultracentrifugation of the cell extracts prior to purification on the affinity columns, was solubilised in SDS-sample buffer and analysed by SDS-PAGE for the presence of recombinant protein. A substantial amount of recombinant protein was still contained within the pellet, giving further explanation for the relatively low yield of purified recombinant protein.

To confirm that the three bacterially expressed and column purified proteins were indeed fragments of caspase 2, a column purified sample of each recombinant caspase

fragment was further purified and separated from bacterial proteins by reverse-phase HPLC (High performance liquid chromatography) (HPLC chromatograms not shown). Column purified recombinant caspase 2 fragments 1 and 2 provided a distinct elution profile and the protein fractions, corresponding in elution time to the predicted molecular weight of fragments 1 and 2, were collected and subjected to NH_2-terminal amino acid sequencing (chromatogram reports not shown). The HPLC profile of column purified recombinant caspase 2 samples further established the presence of both low and high molecular weight contaminating proteins, probably of bacterial origin, in the affinity column purified recombinant caspase 2 preparations.

In the case of the large subunit fragment 3, proteins, most likely of bacterial origin, coeluted of the HPLC column at approximately the same time at which the predicted size recombinant fragment 3 should have eluted and no single prominent fragment 3 protein peak was initially obtained. The HPLC elution profile of larger molecular weight proteins, particularly the 22kDa protein, contained within the fragment 3 protein preparation, which would have eluted later off the HPLC column were not analysed. HPLC fractions from two additional runs were pooled and the amino acid sequencing reaction was repeated in order to determine the NH_2-terminal fragment 3 sequence. This showed that the recombinant caspase 2 fragment 3 band was present in very small quantities within the fragment 3 column purified sample and corresponded to the weak 15.5kDa protein band and not the prominent 22kDa protein.

The NH_2-terminal 10 amino acids of all three recombinant proteins, corresponding in HPLC elution profile to the predicted molecular weight of the three caspase fragments, were thus identified as the correct NH_2-terminal amino acids sequences of caspase 2 (Table 3.2.).

	10 NH_2-terminal amino acids(single letter code)
fragment 1 (prodomain)	MHPHHQETLK
fragment 2 (small subunit)	METDRGVDQQ
fragment 3 (large subunit)	MGPVCLQVKP

Table 3.2. NH_2-terminal amino acid sequencing of three recombinant caspase 2 polypeptides

In order to determine the exact molecular weight and thus confirm the identity of the three recombinant polypeptides, each column purified recombinant caspase 2 fragment was analysed by mass spectrum photometry. Initial mass spectrum photometry results with the recombinant polypeptides showed non-quantitatively the presence of a variety of unidentified proteins present in the Ni^{2+} affinity column eluate fraction and thus further confirmed the impurity of the recombinant polypeptides eluted of the column. Recombinant caspase 2 fragments were thus column purified twice prior to analysis by mass spectrophotometry (Figure 3.7.).

Caspase 2 fragment 1 with a predicted molecular weight of 11974 Dalton most likely corresponded to one of the four proteins found with a molecular mass range of 10187-10838 Dalton, most likely reduced in molecular mass due to decomposition (Figure 3.7.A). Other smaller proteins were found to be present in the sample, and may represent further fragments of the recombinant protein or contaminating bacterial proteins.

Caspase 2 fragment 2 with a predicted molecular weight of 13710.5 Dalton most likely corresponded to the detected protein of 13997.7 Dalton (Figure 3.7.B). The difference between predicted and detected molecular weight was most likely due to inaccurate calibration of the mass spectrum photometer. Similar to the fragment 1 sample, a variety of other unidentified proteins were detected in the fragment 2 sample.

Caspase 2 fragment 3, however, with a predicted molecular weight of 15400 Dalton could not be identified in any of the scans. Instead a protein of 23329.4 Dalton, detected also with a double charge as 11673.6 Dalton protein, was most prominent. This protein most likely corresponded to the 22kDa protein detected by SDS-PAGE. Larger amounts and a less contaminated sample of recombinant fragment 3 would be required to confirm the exact molecular mass and the identity of fragment 3.

The mass spectrometry thus confirmed the presence of fragment 1 and 2 polypeptides of the predicted molecular weight, whereas fragment 3 was undetectable and thus appeared not to be contained within the protein sample analysed.

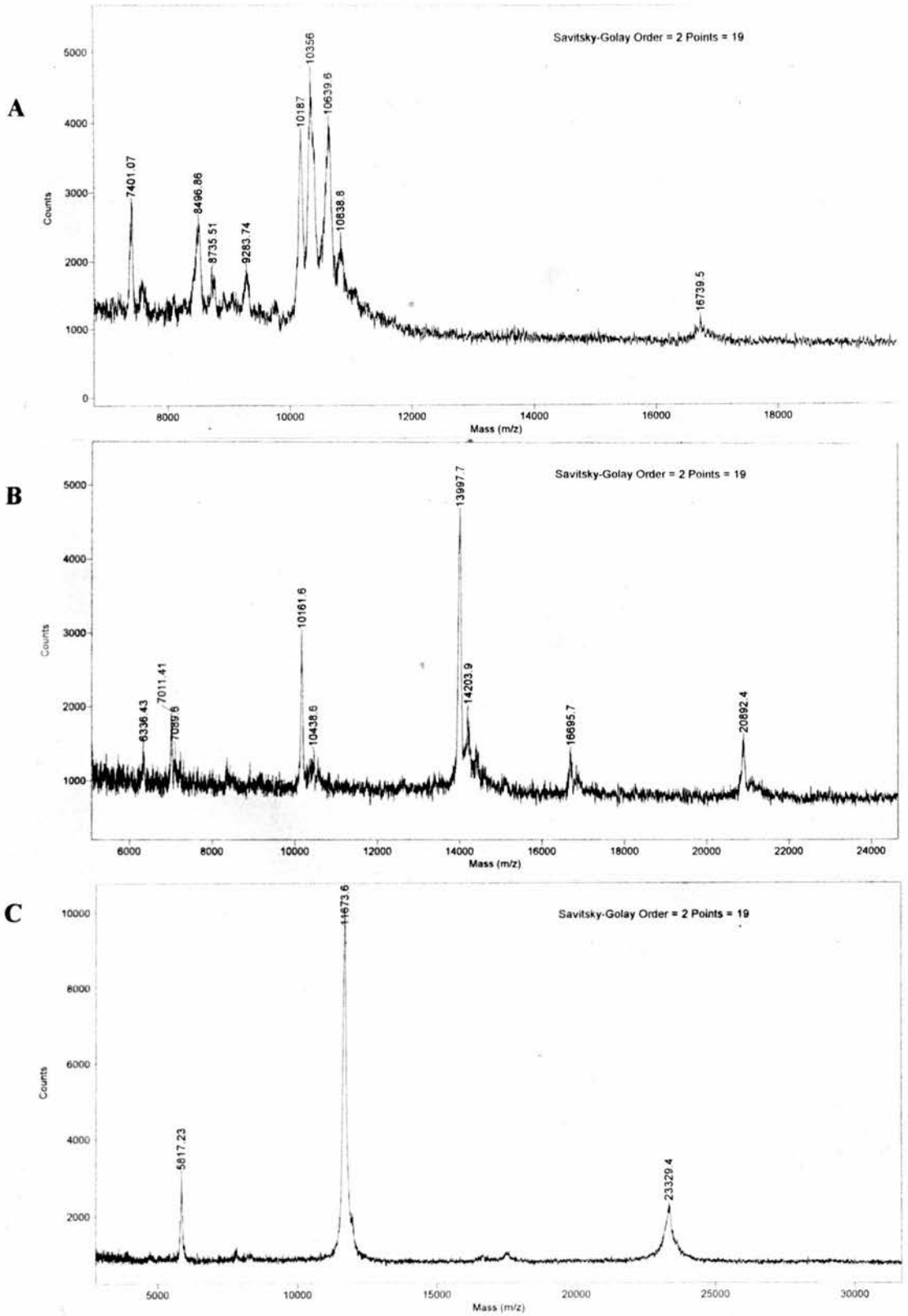


Figure 3.7. Mass spectrum of the column purified recombinant caspase 2 fragments 1-3. A) Mass spectrum column purified fragment 1. Accelerating Voltage: 26250; Low Mass Gate: on; Negative Ions: off; Laser: 905; Scans Averaged: 3; Pressure: 1.88e-07. **B)** Mass spectrum column purified fragment 2. Accelerating Voltage: 26250; Low Mass Gate: on; Negative Ions: off; Laser: 800; Scans Averaged: 2; Pressure: 1.31e-07. **C)** Mass spectrum column purified fragment 3. Accelerating Voltage: 26250; Low Mass Gate: on; Negative Ions: off; Laser: 608; Scans Averaged: 4; Pressure: 1.19e-07.

In summary, all three methods, SDS-PAGE analysis, HPLC, amino acid sequencing and mass spectrometry established the identity of recombinant fragment 1 and 2 as caspase 2 fragments present in high amounts in the eluate fraction, albeit with some additional contaminating proteins. The eluate fraction of cultures expressing fragment 3, however, contained only minimal amounts of the recombinant caspase 2 protein with the majority of protein being contaminating bacterial proteins.

3.2. CHARACTERISATION OF POLYCLONAL ANTIBODIES

The preceding results indicated that recombinant caspase 2 protein fragments 1 and 2 were obtained from bacterial cultures successfully and in sufficient quantities The successful purification of fragment 3 was in substantial doubt and much smaller quantities of the protein were obtained. All three preparations of recombinant proteins were used as antigens, however, for the immunisation of six rabbits and three different polyclonal antisera were generated. The six rabbits (two for each recombinant caspase 2 fragment) were immunised with single column-purified protein fractions according to the following the rabbit immunisation scheme (Table 3.3.).

Caspase 2 fragment 1 Rabbits 16AB + 68AB		**Caspase 2 fragment 2 Rabbits BX03 + BX08**		**Caspase 2 fragment 3 Rabbits 32AB + 90AB**	
prebleed 1		prebleed 1		prebleed 1	
prebleed 2		prebleed 2		prebleed 2	
immunisation 1	(time 0)	immunisation 1	(time 0)	immunisation 1	(time 0)
testbleed 1	(2 wks)	testbleed 1	(2 wks)	testbleed 1	(2 wks)
testbleed 2	(4 wks)	testbleed 2	(4 wks)	testbleed 2	(4 wks)
testbleed 3	(6 wks)	testbleed 3	(6 wks)	testbleed 3	(6 wks)
immunisation 2	(8 wks)	immunisation 2	(8 wks)	immunisation 2	(8 wks)
testbleed 4	(10 wks)	testbleed 4	(10 wks)	testbleed 4	(10 wks)
testbleed 5	(12 wks)	testbleed 5	(12 wks)	testbleed 5	(12 wks)
testbleed 6	(14 wks)	testbleed 6	(14 wks)	testbleed 6	(14 wks)
immunisation 3	(9 month)	immunisation 3	(9 month)		
testbleed 7	(4 wks)	testbleed 7	(4 wks)		

Table 3.3. Rabbit immunisation scheme for polyclonal anti-caspase 2 antibodies. Immunisation 3 at indicated month after initial immunisation for rabbits 68AB and BX08 only; wks = weeks.

Following the first and second immunisation with recombinant column purified polypeptides 1, 2 and 3, the six rabbits were testbled every two weeks and the testbleed sera, initially only of the rabbits immunised with caspase 2 fragment 1 and 2, were analysed for anti-recombinant caspase 2 immuneresponse by Western blotting. Antisera to the caspase 2 fragment 3 preparations were obtained 3 months after those initial experiments.

3.2.1. Determination of whole serum antibody titre against recombinant caspase 2 fragments

In order to analyse whether the rabbits had shown an immuneresponse to the recombinant caspase 2 fragments and to determine the polyclonal antibody binding affinity for the recombinant polypeptides a series of whole testbleed sera dilutions were tested against a constant amount of recombinant polypeptide using a multi-well blotter (Figure 3.8.).

Testbleed sera from rabbits 68AB and 16AB (Figure 3.8.A) showed a strong immune response to the caspase 2 polypeptide fragment 1 and were able to bind to fragment 1 at 1:1000 dilution, whereas prebleed sera showed no immunoreactivity against the caspase 2 polypeptide. Testbleed antisera from rabbits BX03 and BX08 (Figure 3.8.B) were equally able to detect recombinant fragment 2 at 1:1000 dilution while prebleed sera were unable to detect the recombinant polypeptide. Testbleed sera 16AB, 68AB and BX08 further detected multiple higher molecular weight bands on the Western blots, which correspond in size to dimer- and multimer-formations of the recombinant polypeptides. These dimers and multimers appear to be formed at high recombinant protein concentrations. When substantially less recombinant protein (50μg in comparison to 500μg) was run on SDS-PAGE and subsequently analysed by Western blotting no higher molecular weight bands were detected, indicating that the recombinant protein was fully denatured (data not shown).

In view of these results sera from rabbits 32AB and 90AB, immunised with the attempted fragment 3 preparation, were analysed on ten times less recombinant polypeptide (50μg). At 1:500 dilution the testbleed sera bound to many proteins, but in particular a 22kDa protein band (Figure 3.8.C). Both rabbits showed, in comparison to rabbits immunised with fragment 1 and 2, a later immune response to the antigen. These results suggest that the poor quality of the immunogen did not give rise to fragment 3 antibodies, and ELISA data finally confirmed this conclusion (see chapter 3.2.3.).

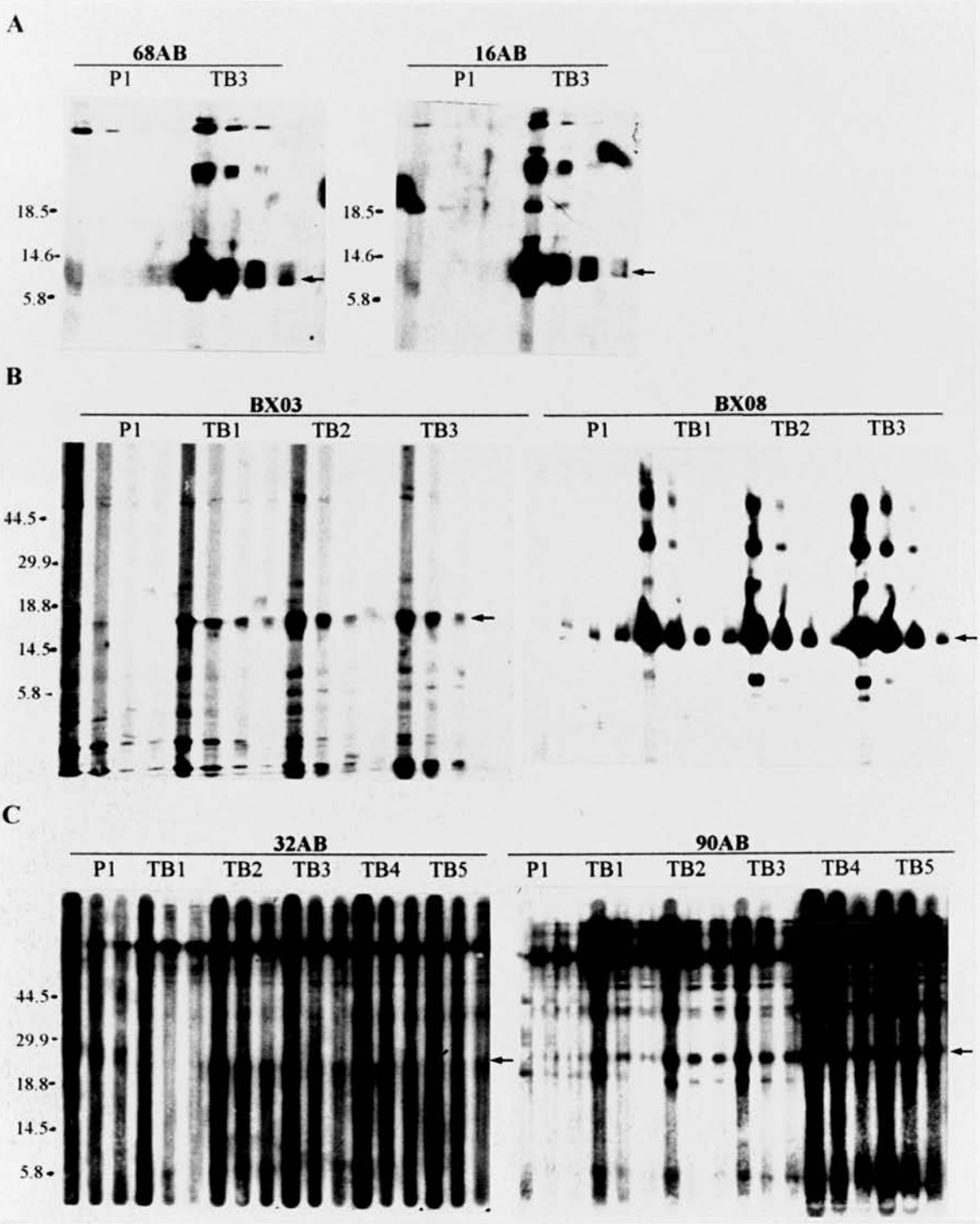


Figure 3.8. Immunoblot analysis of polyclonal antibody affinity to recombinant caspase 2 fragments. 15% SDS-PAGE minigel, transferred to nitrocellulose and probed with serial dilutions of non-purified polyclonal anti-caspase 2 testbleed sera (TB) and prebleed sera (PB). Molecular weight standards in kDa. The position of each recombinant polypeptide is indicated by an arrow. **A**) 500μg of fragment 1 detected with serial dilutions of fragment 1 polyclonal antisera, rabbit 16AB and 68AB (dilutions from left to right: 1:30; 1:100; 1:500; 1:1000). **B**) 500μg of fragment 2 detected with serial dilutions of fragment 2 polyclonal antisera, rabbit BX03 and BX08 (dilutions from left to right: 1:30; 1:100; 1:1000); visible bands in the prebleed wells of rabbit BX08 were due to leakage of testbleed sera after disassembly of the multi-well blotter. **C**) 50μg of fragment 3 detected with serial dilutions of fragment 3 polyclonal antisera, rabbit 32AB and 90AB (dilutions from left to right: 1:100; 1:250; 1:500).

The antibody affinity to the recombinant fragment 1 and 2 antigen was further characterised by testing the antibody at the previously determined optimal dilution on varying amounts of recombinant immunogen. These experiments aimed to give an indication of the minimum level of detection of endogenous caspase 2 in whole cell lysates by these antibodies, as both the expression level and stability of caspase 2 in cells is unknown. (Figure 3.9.)

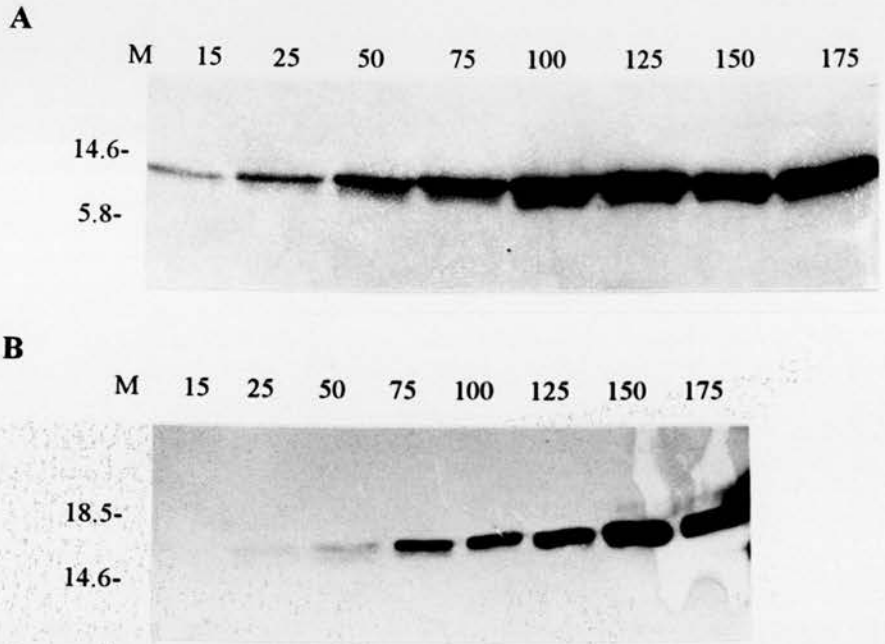


Figure 3.9. Immunoblot analysis of antibody affinity to recombinant caspase 2 fragments. Whole polyclonal antiserum at 1:1000 dilution tested against various concentrations of recombinant polypeptide; 15-175ng/lane run on 15% SDS-PAGE minigel. M: molecular mass marker in kDa. **A)** caspase 2 fragment 1, whole testbleed serum 68AB; **B)** caspase 2 fragment 2, whole testbleed serum BX08.

Anti- fragment 1 antibody 68AB detected down to 15ng of recombinant polypeptide fragment 1 at a 1:1000 dilution. 25ng was optimal for detection at the given dilution. Anti-fragment 2 antibody BX08 was able to detect 25ng of recombinant fragment 2 at 1:1000 dilution, although,. 50ng was optimal. Both anti- fragment 1 and 2 antibodies thus seemed to be promising tools in the detection of endogenous caspase 2.

3.2.2. Determination of purified antibody titre against recombinant caspase 2 fragments

The initial Western blots were carried out with whole serum and confirmed a strong immune response of the rabbits to recombinant caspase 2 antigens (fragment 1 and 2). To determine both the immunoglobulin class and the actual amounts of the anti-caspase 2 polyclonal antibodies within the whole serum, testbleed antisera from individual rabbits were pooled (T2-T6 for fragment 1 and 2 antisera; T4-T6 for fragment 3 antisera) and the IgG fractions were purified by Protein A and G column affinity purification. Polyclonal antibody affinity against recombinant polypeptide was subsequently determined with purified IgG antibodies from rabbits 68AB (fragment 1) and BX08 (fragment 2) (Figure 3.10.).

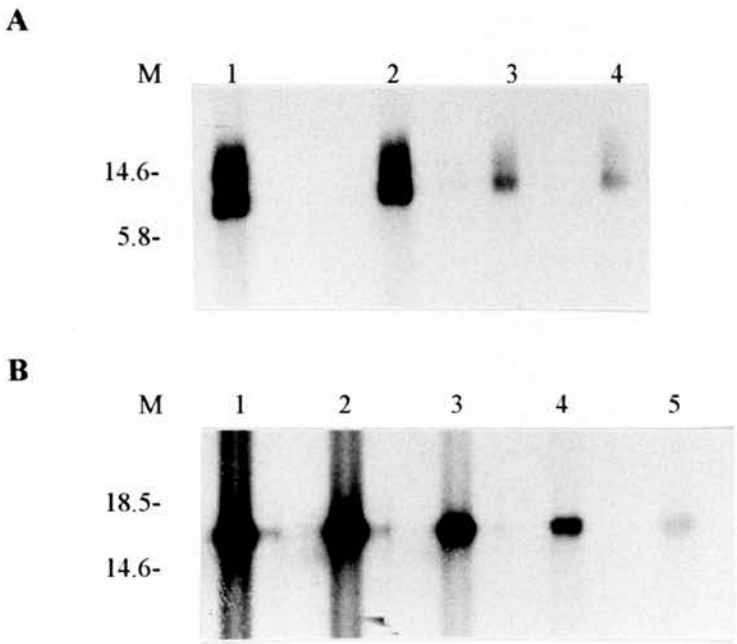


Figure 3.10. Antibody affinity determination of purified antisera by Western blotting analysis. 7μg of recombinant caspase 2 fragments were run on an analytical comb on 15% SDS-PAGE minigel, probed on a multi-well blotter with serial dilutions of purified (IgG fraction) polyclonal antiserum (total protein in μg). M: molecular weight markers in kDa. **A)** varying amounts of purified 68AB polyclonal antiserum tested against recombinant fragment 1; *lane 1:* 100μg; *lane 2*: 75μg; *lane 3:* 50μg; *lane 4:* 25μg **B)** varying amounts of purified BX08 polyclonal antiserum tested against recombinant fragment 2. *lane 1:* 100μg; *lane 2:* 75μg; *lane 3:* 50μg; *lane 4*: 25μg; *lane 5:* 15μg.

Purified IgG fractions of antisera 68AB and BX08 maintained a very high affinity towards the recombinant polypeptide antigen and were able to bind to the recombinant protein when blots were probed with 20μg of 68AB (Figure 3.10. A, lane 4) and 10μg of BX08 (Figure 3.10. B, lane 5). This suggests that both polyclonal anti-caspase 2 antibodies are IgG class molecules.

3.2.3. Antibody specificity testing by Western blotting and ELISA

In experiments to define specificity of the polyclonal antibodies two questions were addressed. First can cross-reactivity between anti-fragment 1 and anti-fragment 2 antibodies be excluded? This is important as the antibodies were designed to discriminate between proenzyme (fragment 1 antibody) and the small subunit (fragment 2 antibody) (see also chapter 3.1.1.) and cross-reactivity, particularly of anti-fragment 1 antibody with other areas of caspase 2, would have destroyed this. Second, the antibodies were tested for reactivity against recombinant subunits from a variety of other caspases. The intention here was to determine the usefulness of the anti-caspase 2 antibodies to distinguish caspase 2 from other members of the caspase family. The first question was approached by Western blotting, using unpurified antisera. Antibodies to fragment 1 were tested for their ability to detect and therefore cross-react with recombinant fragment 2 and antibodies to fragment 2 were tested for their ability to detect and therefore cross-react with recombinant fragment 1 (Figure 3.11.).

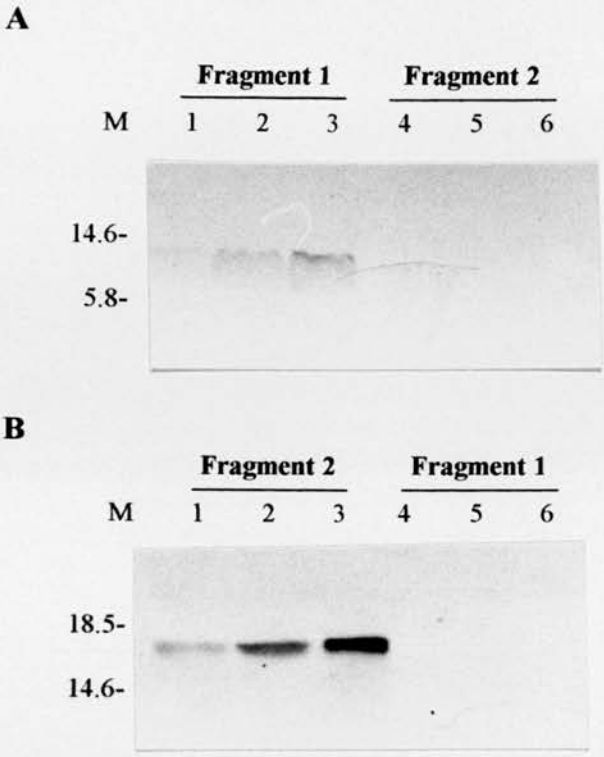


Figure 3.11. Immunoblot analysis of whole serum antibody cross reactivity. Indicated amounts of recombinant caspase 2 fragments were run on 15% SDS-PAGE minigel. M: molecular weight markers are indicated in kDa on the left of each blot. **A)** Fragment 1 antiserum 68 AB (testbleed 5) at 1:1000 dilution. *Lane 1:* 25ng; *lane 2:* 50ng; *lane 3:* 75ng; *lane 4:* 25ng; *lane 5:* 50ng; *lane 6:* 75ng. **B)** Fragment 2 antiserum BX08 (testbleed 6) at 1:1000 dilution. *Lane 1:* 25ng; *lane 2:* 50ng; *lane 3:* 75ng ; *lane 4:* 25ng; *lane 5:* 50ng; *lane 6:* 75ng .

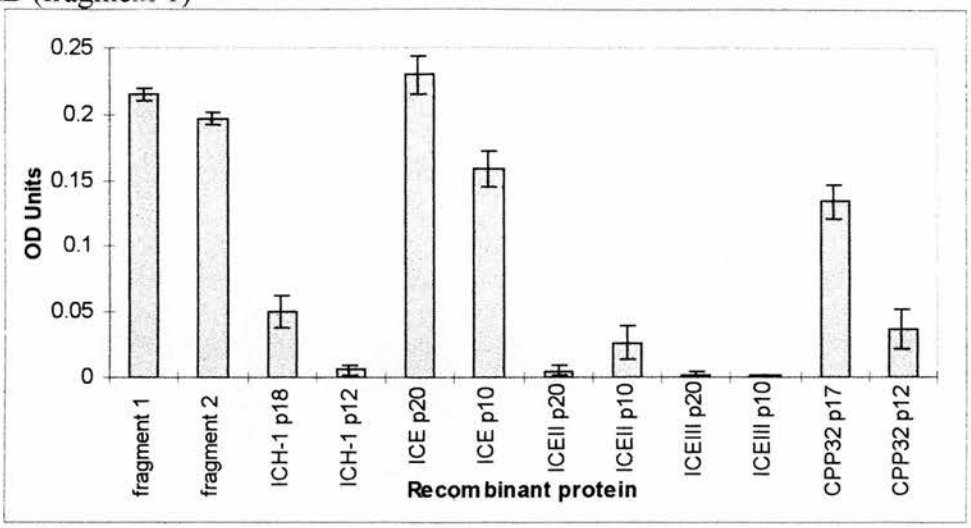
Both antibodies bound to the recombinant polypeptide fragment they were raised against but failed to detect the other recombinant polypeptide thus showing no cross reactivity within caspase 2.

The second question of cross-reactivity of the antibodies with other recombinant caspase family members was tested by ELISA. Recombinant caspase subunits were a kind gift from D.W. Nicholson and include large and small subunits of caspase 1 (ICE), caspase 2 (ICH-1), caspase 3 (CPP32), caspase 4 (ICE_{rel}-II) and caspase 5 (ICE_{rel}-III). Each polyclonal antibody was expected to bind specifically with high affinity to its recombinant fragment antigen, whereas prebleed sera at the same dilution should have no affinity towards the same amount of recombinant polypeptide fragment. Additionally the antibodies to fragment 2 were expected to bind to the

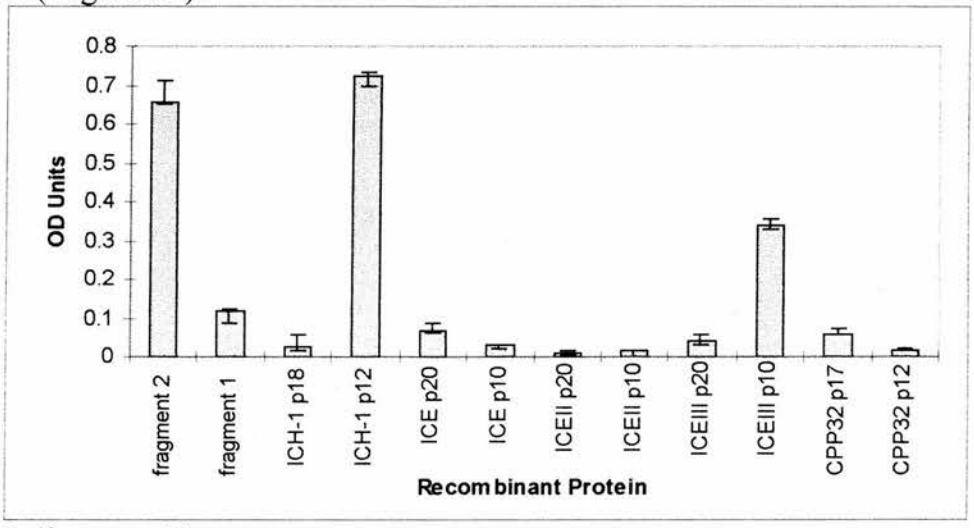
recombinant caspase 2 small subunit and antibodies to fragment 3 were expected to bind to the recombinant caspase 2 large subunit.

In initial ELISAs the affinity of the antibodies for recombinant caspase 2 fragment preparations was established by testing various dilutions of 68AB (fragment 1), BX08 (fragment 2), 32AB (fragment 3) prebleed and testbleed sera against serial dilutions of the immunogen (data not shown). Preliminary ELISA results for fragment 1 antibody 68AB determined the optimal dilution of 1:100 detecting 75ng of bound fragment 1. The binding affinity of 68AB towards the recombinant polypeptide fragment 1 was, however, very low with similar OD values reached when tested for binding to fragment 2. ELISAs for the fragment 2 antibody BX08 suggested an optimal dilution of 1:250 binding to 300-500ng of fragment 2 and fragment 3 antibody 32AB showed an optimal dilution of 1:500 detecting 50ng. At those dilutions the testbleed sera specifically bound to the recombinant protein samples with no cross-reactivity to other caspase 2 fragments and the OD values of prebleed sera were similar to the blank OD values. The antibodies were then tested at the determined optimal concentrations for their affinity for the entire recombinant small subunit of caspase 2 and for other recombinant caspases (Figure 3.12.).

A, 68AB (fragment 1)



B, BX08 (fragment 2)



C, 32AB (fragment 3)

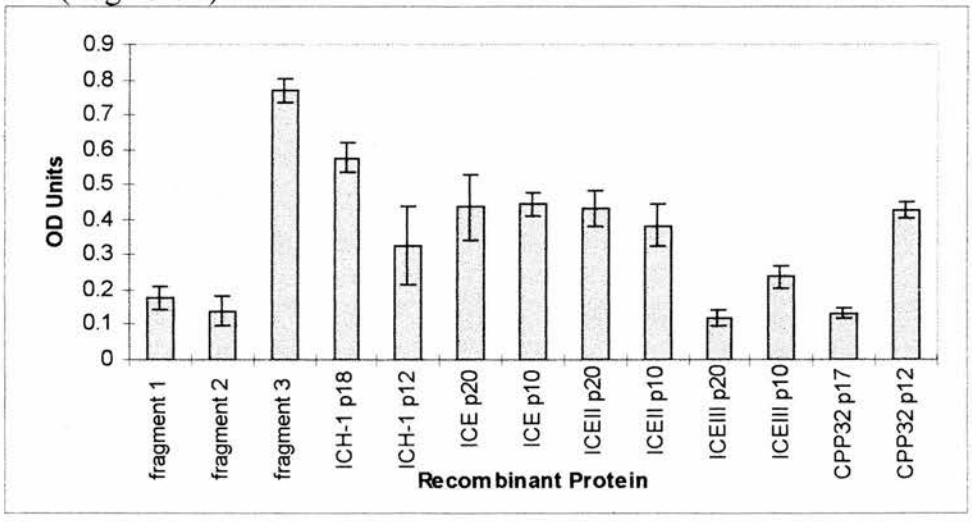


Figure 3.12. ELISA antibody cross-reactivity testing. A) 68AB polyclonal antiserum at a dilution of 1:100 (purified stock 1mg/ml), ELISA plate coated with 75ng recombinant protein; **B)** BX08 at 1:250 dilution (purified stock 1mg/ml), plate coated with 300ng recombinant protein; **C)** 32AB at 1:500 dilution (purified stock 1mg/ml), plate coated with 100ng recombinant protein.

Polyclonal antibody 68AB bound to recombinant fragment 1 with very low affinity with OD values similar to fragment 2 and was also able to detect caspase 1 (ICE) large and small subunit and caspase 3 (CPP32) large subunit (Figure 3.12.A). Binding of 68AB to both fragment 1 and fragment 2 was abolished when the antibody was pre-incubated with recombinant caspase 2 fragment 2 (data not shown), confirming that the observed binding of the antibody was most likely background binding to bacterial proteins. ELISA analysis with purified 68AB fragment 1 antibody thus showed that the antibody is unsuitable for ELISA. A potential explanation for this unsuitability of the antibody 68AB could reside in the epitope that the antiserum recognises. ELISA is carried out under non-denaturing conditions allowing the reformation of some secondary structures including disulphide-bridges. Should the antiserum mainly recognise a linear epitope, fully denatured in SDS-PAGE, the very low binding of the antibody in ELISA could be explained.

Purified 32AB polyclonal antibody bound with high affinity to all caspase subunits tested, particularly caspase 1 large and small subunit, caspase 4 large and small subunit and caspase 3 small subunit (Figure 3.12.C), further confirming the unsuitability of the antibody for further experiments. Like the antigen to which 32AB was raised, the caspase subunits tested for cross-reactivity are also bacterially expressed recombinant proteins of unknown purity and are, therefore, likely to contain some bacterial proteins. What appeared to be wide cross-reactivity of the 32AB antibody with other caspase subunits is thus most likely antibody binding to bacterial proteins rather than actual caspase subunits.

Purified BX08 antibody raised against fragment 2 showed high specificity towards caspase 2 small subunit and caspase 2 fragment 2 with an almost identical signal strength (Figure 3.12.B). The antibody showed no significantly cross-reactivity with caspase 2 large subunit or any of the other four caspase subunits tested, with the exception of the small subunit of caspase 4 to which it showed a low affinity. When

this antibody was pre-absorbed with recombinant caspase 2 fragment 1, the binding to recombinant fragment 1 was reduced to background values, while a high affinity was retained towards the immunogen fragment 2 (Figure 3.13.). Preabsorption thus further confirmed the specificity of the COOH-terminal anti-caspase 2 antibody to recombinant caspase 2 small subunit.

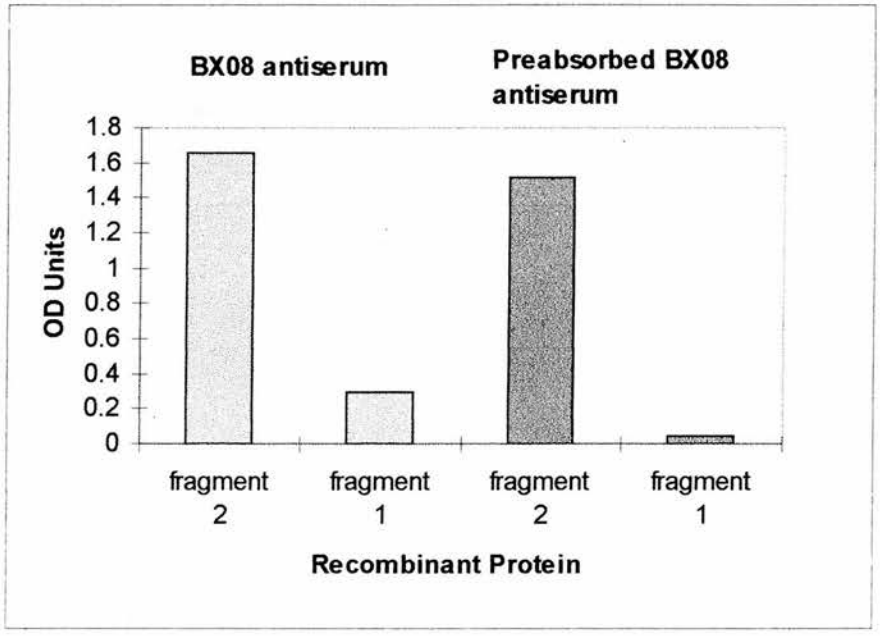


Figure 3.13. Comparative ELISA of absorbed and non-absorbed BX08 antiserum. ELISA plate coated with 300ng recombinant caspase fragment, antibody at 1:250 dilution. OD measured at 450nm.

3.2.4. Endogenous caspase 2 detection in whole cell extracts of three human cell lines

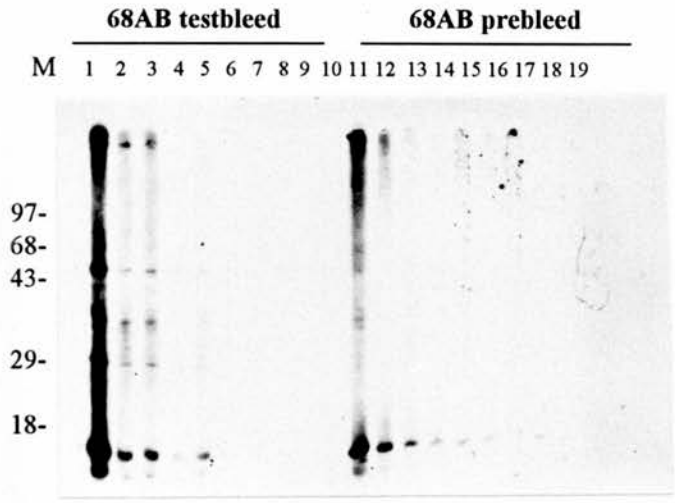
Following the demonstration of antibody affinity and specificity, partly by Western blotting analysis and ELISA, for recombinant subunits and fragments of caspase 2, the polyclonal antisera were tested for their ability to detect endogenous procaspase 2 in whole cell extracts of three human cell lines. Fragment 1 and 2 antisera (both purified IgG fractions and unpurified whole sera) together with the preimmune sera as negative controls were tested at various concentration (1:1000; 1:500; 1:250; 1:200; 1:100; 1:50) for their ability to detect caspase 2 in whole cell protein extracts of HeLa and Jurkat cells, reported to express high levels of caspase 2 (Wang *et al*, 1994).

In Western blotting analysis preimmune and testbleed sera of rabbits 16AB and 68AB (prodomain/fragment 1 antibodies) and rabbits BX03 and BX08 (small subunit/fragment 2 antibodies) at all concentrations tested failed to detect a protein of 48kDa corresponding to the predicted molecular weight of procaspase 2 (data not shown). Prebleed and testbleed sera of rabbits 16AB and 68AB, however, strongly bound to two proteins of approximately 29kDa and 18kDa while prebleed and testbleed sera from rabbits rabbits BX03 and BX08 detected an 18kDa protein band. Upon long exposure of the film to the membrane at 1:100 and 1:50 dilution prebleed and testbleed sera of both antibodies detected a variety of high (>50kDa) and low (<50kDa) molecular weight additional protein bands and a band with similar mobility to caspase 2 in HeLa and Jurkat cell extracts. Comparison of preimmune and testbleed antibody binding to a 48Kda protein corresponding to caspase 2 showed no clear difference in binding due to the presence of additional nonspecific bands, which migrated close to the that of full-length caspase 2. A commercial anti-caspase 2 monoclonal antibody at 1:1000 dilution used as a positive control, however, bound specifically with no nonspecific background binding to a protein of about 46kDa, approximately corresponding to the predicted molecular weight of caspase 2 (see chapter 3.3.1.).

These Western blotting results showing high cross-reactivity with binding of the polyclonal anti-caspase 2 antibodies to non-specific bands and low affinity to caspase 2 to caspase 2 contrasted so sharply with the previously obtained specific binding to recombinant peptide in Western blots, that it seemed possible that the antibody preparations had lost their activity. Accordingly the Western blotting was repeated with the HeLa extract "spiked" with 500μg recombinant polypeptides, run on a large SDS-PAGE gel with an analytical comb (data not shown). Purified and non-purified fragment 1 antibodies 16AB and 68AB at 1:50 dilution were able to detect the monomers and multimers of recombinant fragment 1 polypeptide (500μg) amongst HeLa proteins and also detected other nonspecific protein bands. In contrast, negative control purified and non-purified 68AB prebleed sera bound to the same HeLa proteins but were unable to detect recombinant fragment 1 added to HeLa extracts.

This experiment confirmed that the disappointing initial failure to detect caspase 2 in protein extracts by Western blotting was not due merely to inactivation of the antibodies on storage. Accordingly Western blotting was repeated under altered conditions chosen to optimise binding of lower-affinity antibodies to caspase 2. Thus the primary antibody incubation time was extended, longer washes and an immunopure secondary antibody were applied. Titrations of both prodomain and small subunit antibodies were tested for their ability to detect caspase 2 in HeLa and Jurkat whole cell extracts (Figures 3.14. and 3.15.). In contrast to earlier results, this altered protocol produced dramatically different results.

A



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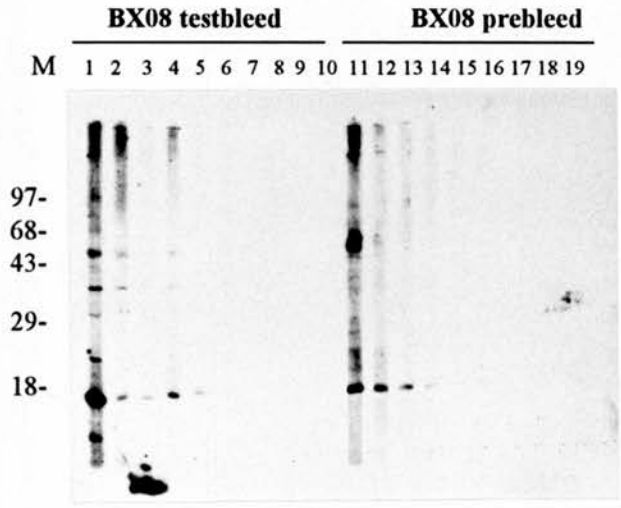

Figure 3.14. Immunoblot analysis of caspase 2 detection in HeLa whole cell extract. 12.5% SDS-PAGE minigel, 200µg whole cell extract run on analytical comb, purified 68AB and BX08 prebleed and testbleed sera at 1mg/ml, titration as indicated; M: molecular weight standards in kDa. **A** and **B**, *lane 1:* 1:50; *lane 2:* 1:100; *lane 3:* 1:200; *lane 4:* 1:300; *lane 5:* 1:400; *lane 6:* 1:500; *lane 7:* 1:750; *lane 8:* 1:1000; lane 9: 1:2000; *lane 10:* no primary antibody; *lane 11:* 1:50; *lane 12:* 1:100; *lane 13:* 1:200; *lane 14:* 1:300; *lane 15:* 1:400; *lane 16:* 1:500; *lane 17:* 1:750; *lane 18:* 1:1000; *lane 19:* 1:2000.

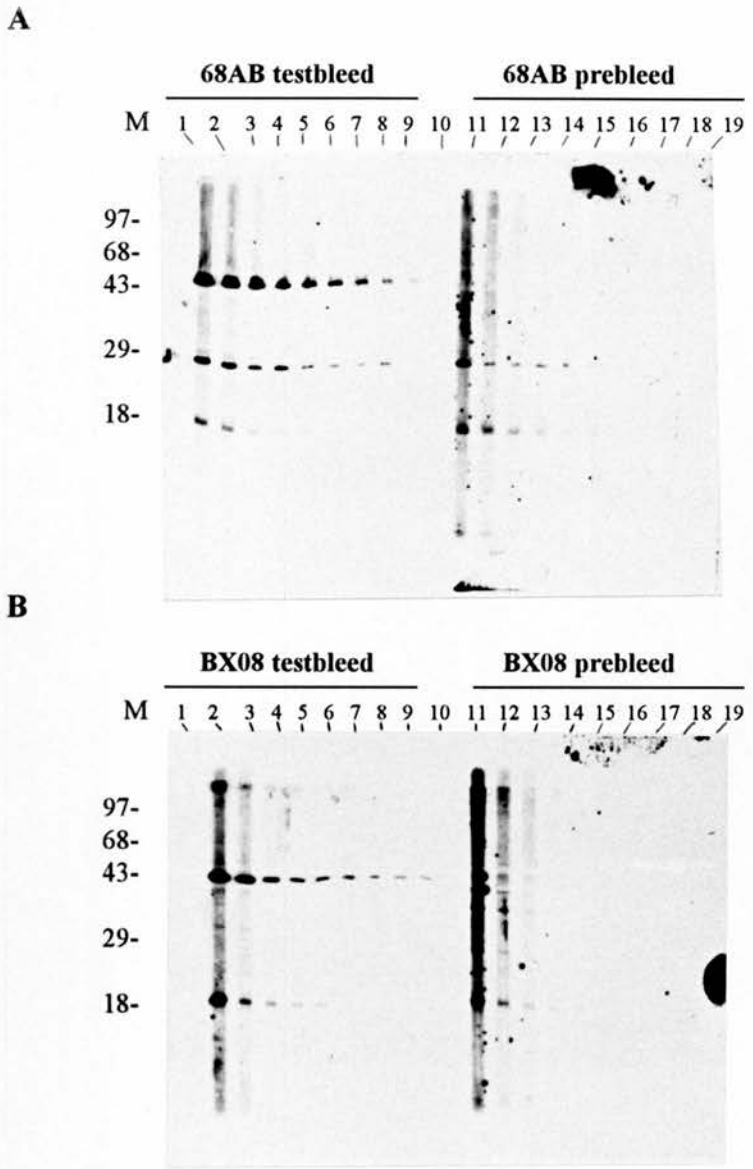

Figure 3.15. Immunoblot analysis of caspase 2 detection in Jurkat whole cell extract. 12.5% SDS-PAGE minigel, 200µg whole cell extract run on analytical comb, purified 68AB and BX08 prebleed and testbleed sera at 1mg/ml, titration as indicated; M: molecular weight standards in kDa. **A** and **B**, *lane 1:* 1:50; *lane 2:* 1:100; *lane 3*: 1:200; *lane 4:* 1:300; *lane 5:* 1:400; *lane 6:* 1:500; *lane 7:* 1:750; *lane 8* 1:1000; lane 9: 1:2000; *lane 10:* no primary antibody; *lane 11:* 1:50; *lane 12:* 1:100; *lane 13:* 1:200; *lane 14:* 1:300; *lane 15:* 1:400; *lane 16:* 1:500; *lane 17:* 1:750; *lane 18:* 1:1000; *lane 19:* 1:2000.

Both polyclonal anti-caspase 2 antibodies were able to detect endogenous caspase 2 with similar affinity in human Hela and Jurkat cell extracts. 68AB and BX08 testbleed sera detected a 48kDa protein in HeLa cell extracts at 1:50; 1:100; 1:200 and 1:300

dilution, while prebleed sera at the same dilutions failed to detect a protein of the same molecular weight (Figure 3.14 A and B). 68AB and BX08 testbleed and prebleed sera both detected an additional four lower molecular weight proteins of approximately 35kDa, 29kDa, 23kDa and 18kDa. Both anti-caspase 2 antisera detected caspase 2 in Jurkat extracts in up to 1:2000 dilution, indicating higher expression levels of caspase 2 in Jurkat cells. Prebleed and testbleed sera of antibody 68AB, however, also bound to two lower molecular weight proteins in Jurkat extracts of approximately 29kDa and 18kDa, whereas prebleed and testbleed sera of anti-caspase 2 antibody BX08 detected only the lower molecular weight 18kDa bands. Binding to these nonspecific bands decreased at higher dilutions and was undetectable at the 1:2000 for prodomain antibody 68Ab and 1:500 dilution for small subunit antibody BX08, dilutions at which caspase 2 was still weakly detected by both testbleed sera. Extended incubation with the primary antibody, a higher purified secondary antibody, increased protein amounts in the blocking buffer and carefully timed washing steps thus proved to be a requirement to decrease background binding and ensure caspase 2 detection with both antibodies.

3.2.5. Endogenous caspase 2 detection in human tissue extracts

Additional to the reported expression of caspase 2 in a variety of human cell lines, Northern blotting data has shown expression of a *caspase 2* mRNA in a variety of human tissues (Wang *et al*, 1994). In order to characterise the function of caspase 2, soluble tissue extracts were prepared of 11 human tissues, including three different lung cases and the caspase 2 protein expression pattern was analysed by Western blotting using the fragment 1 and 2 anti-caspase 2 polyclonal antibodies (Figure 3.16).

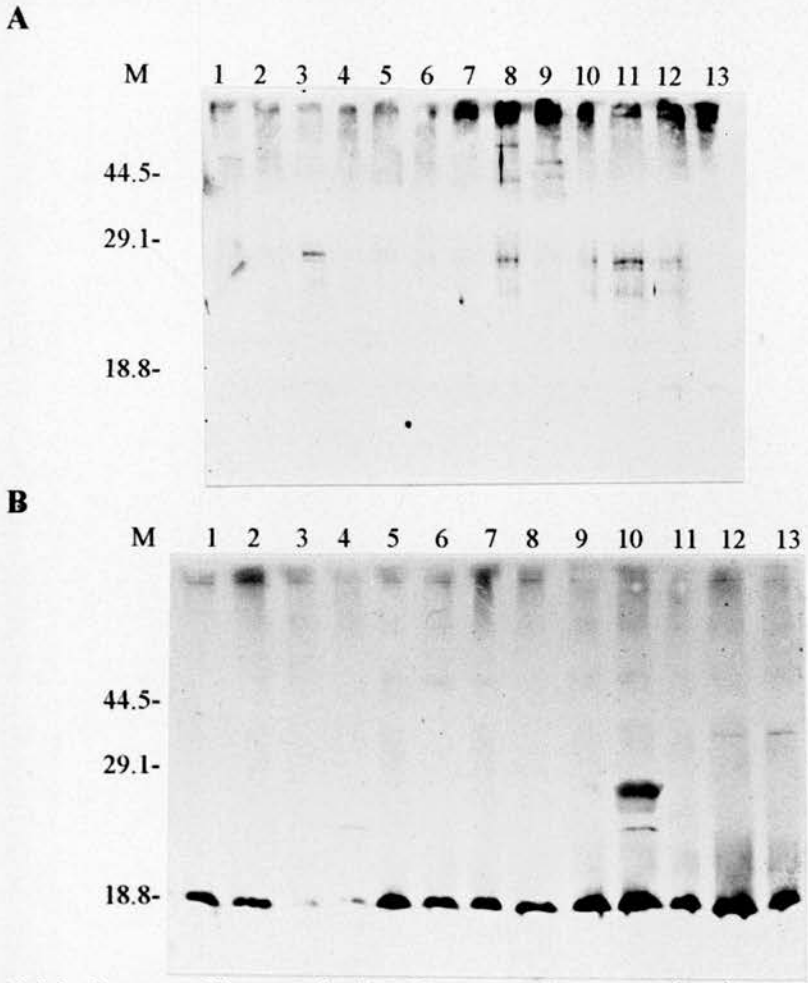

Figure 3.16. Immunoblot analysis of caspase 2 expression in 11 human tissues. 30µg sample/lane run on 12.5% SDS-PAGE minigel, transferred to nitrocellulose and probed with purified polyclonal antisera at 1:100 dilution. M: molecular weight standards in kDa. **A)** antibody 68AB; l*ane 1*: cerebrum, *lane 2*: heart, *lane 3:* kidney, *lane 4:* liver, *lane 5:* lung (case 387), *lane 6:* lung (case 388), *lane 7:* lung (case 396), *lane 8*: pancreas, *lane 9*: prostate, *lane 10*: skeletal muscle, *lane 11*: spleen, *lane 12*: small intestine, *lane 13*: testes. **B)** antibody BX08; l*ane 1*: cerebrum, *lane 2*: heart, *lane 3:* kidney, *lane 4:* liver, *lane 5:* lung (case 387), *lane 6:* lung (case 388), *lane 7:* lung (case 396), *lane 8*: pancreas, *lane 9*: prostate, *lane 10*: skeletal muscle, *lane 11*: spleen, *lane 12*: testes, *lane 13*: small intestine.

At antibody dilutions of 1:400 -1:1000, which in previous Western blotting results of human cell lines showed clear binding to caspase 2, both polyclonal caspase 2 antibodies failed to detect a protein of the correct molecular weight (48kDa) while antibody 68AB in particular strongly bound to an approximately 18kDa protein which most likely corresponds to the previously identified protein band in HeLa and Jurkat cells. At a dilution of 1:300 with variability in binding between different blots and very

low affinity, detectable only after long exposure of the film to the blot (approximately 40 min), antibody 68AB bound to a protein of about 48kDa in cerebrum, lung and small intestine protein extracts (Figure 3.16.A), while antibody BX08 detected very faintly a protein of about 48kDa in cerebrum and lung (Figure 3.16.B). Both antibodies detected a nonspecific band at 18kDa. Similar results were obtained with antibodies 16AB and BX03 (data not shown).

The required antibody dilution of 1:300 points to the low affinity of the antibodies, an observation also found in later experiments with other noncommercial anti-caspase 2 polyclonal antibodies (see chapter 3.6.7.). The data, however, also suggests that protein expression levels of procaspase 2 are very low in the human tissues analysed and suggest a potential role of caspase 2 in the brain, lung and small intestine, although it is impossible to infer function merely from protein expression patterns.

3.2.6. Immunocytochemical detection of caspase 2

The ability of the caspase 2 polyclonal antibodies to detect caspase 2 in immunocytochemistry was tested. Cytospins of exponentially growing non-apoptotic human Jurkat, HeLa and H9 were prepared and various concentrations (1:10; 1:20; 1:30; 1:50 and 1:100) of both purified IgG antisera, whole sera and preimmune sera were used in the immunocytochemical detection of cytoplasmic procaspase 2 (Figure 3.17.). Negative control cytospins incubated with secondary antibody only were included in each experiment.

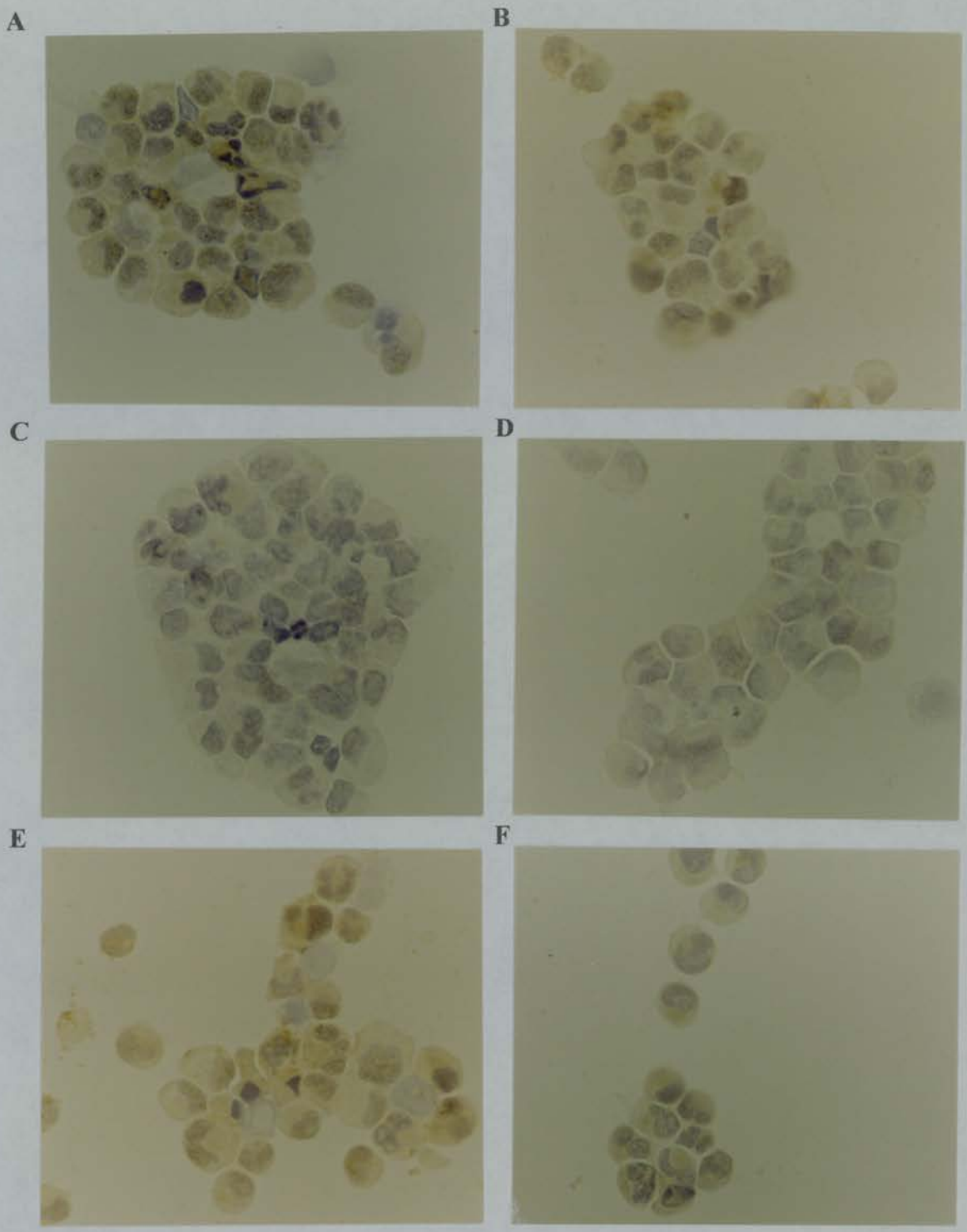

Figure 3.17. Immunocytochemical analysis of caspase 2 expression in H9 cells with polyclonal anti-caspase 2 antibodies. Cytospins H9 cells were incubated with purified polyclonal anti-caspase 2 fragment 1-3 antisera and pre-immune sera at indicated dilutions, secondary swine anti-rabbit HRP coupled antibody (1:100 dilution), visualisation with DAB; magnification 400x. PB = prebleed, TB = testbleed. *Panel A*: TB BX08 1:100; *panel B*: PB BX08 1:100; *panel C*: TB BX08 1:200; *panel D*: PB BX08 1:200; *panel E*: TB 68AB 1:100; *panel F*: PB 68AB 1:100.

No specific staining relative to the prebleed control was observed with any of the antibodies. H9 cytospins probed with purified BX08 testbleed serum at 1:100 dilution showed cytoplasmic immunostaining (Figure 3.17., panel A) with identical staining pattern at the same dilution purified BX08 prebleed sera (Figure 3.17, panel B). This cytoplasmic prebleed staining was much reduced at 1:200 dilution (Figure 3.17. panel D) but testbleed sera at the same dilution also showed no staining (Figure 3.17. panel C). 68AB testbleed/prebleed (panels E and F) gave similar results with further serum dilutions resulting in loss of staining of both test- and prebleeds (data not shown). Immunocytochemical analysis of HeLa and Jurkat cells gave identical results. Both polyclonal antibodies from all four rabbits thus failed to detect caspase 2 in cytospins of all three cell lines which suggests that either both antibodies require a linear denatured epitotope or that the binding to caspase 2 is overshadowed by the nonspecific binding of the antibodies to the 29kDa and 18kDa protein bands. In contrast immunocytochemical experiments with commercial anti-caspase 2 antibodies demonstrated high levels of a cytoplasmic protein in all three human cell lines (see chapter 3.3.3., Figure 3.19.). The polyclonal anti-caspase 2 antibodies were, nevertheless, tested for their ability to detect a product in tissue sections.

3.2.7. Immunohistochemical detection of caspase 2

Immunohistochemical analysis of paraffin embedded human tissues, including colon, liver, pancreas, tonsil and thymus was carried out with both anti-caspase 2 polyclonal antibodies (data not shown). Comparison of both purified and non-purified prebleed and testbleed sera staining showed identical results: none of the polyclonal sera was able to detect caspase 2. These results confirmed the unsuitability of all polyclonal antisera raised against recombinant caspase 2 polypeptides in both the immunohistochemical and immunocytochemical detection of caspase 2. Other non-commercial anti-caspase 2 antibodies do, however, also give very poor results in immunohistochemistry (J.Yuan, personal communication).

In summary, the attempt to raise antibodies to fragment 3 produced no satisfactory reagents, probably because the immunogen was poor in concentration of the appropriate caspase 2 peptide and was heavily contaminated with bacterial proteins. In contrast two antisera, were raised against successfully purified recombinant caspase 2 polypeptides to which the rabbits showed a strong immuneresponse. Using a standard Western blotting protocol both antibodies bound non-specifically to a variety of protein bands potentially including procaspase 2. Under altered conditions designed to enhance binding of low affinity antibodies, both fragment 1 (prodomain) and fragment 2 (small subunit) antibodies successfully detected procaspase 2 in nonapoptotic HeLa and Jurkat cell extracts and bound specifically to procaspase 2, albeit with weak affinity in Western blotting of tissue extracts. Both polyclonal anti-caspase 2 antibodies failed in immmunohistochemical- and immunocytochemical detection of caspase 2 and thus could not be used to investigate its subcellular localisation, relative levels of expression and distribution in tissues. Despite these limitations, the usefulness in Western blotting of the antibodies raised against fragments 1 and 2 permitted their application to the analysis of caspase 2 activation in apoptosis (chapter 3.6.7.).

3.3. CHARACTERISATION OF COMMERCIAL ANTI-CASPASE 2 MONOCLONAL ANTIBODY

3.3.1. Determination of commercial antibody specificity

Whilst the experiments with the fragment 1, 2 and 3 antibodies were proceeding, two anti-caspase 2 antibodies became available commercially, an anti-peptide polyclonal anti-caspase 2 antibody (Santa Cruz Biotechnology) and a recombinant polypeptide anti-caspase 2 monoclonal antibody (Transduction Laboratories) (see Appendix 2, Figure 3. Antigen for caspase 2 polyclonal and monoclonal antibodies). The availability of the commercial antibodies suggested an alternative route to meet the initial aims of this thesis. In order to test these commercial antibodies for their affinity and specificity both antibodies were applied in Western blotting analysis of HeLa extracts for their ability to detect caspase 2 proenzyme (Figure 3.18.).

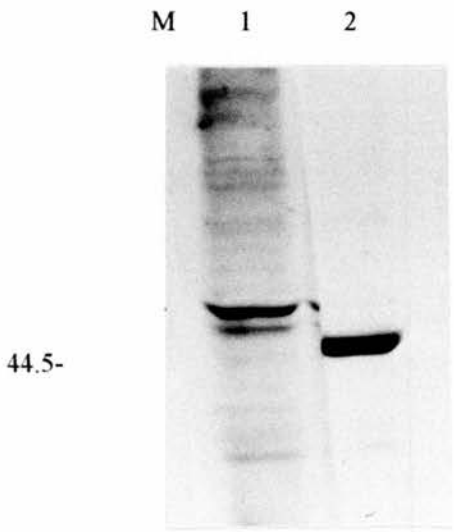

Figure 3.18. Immunoblot analysis of caspase 2 expression in HeLa cells. HeLa whole cell protein extracts (100μg/lane) run on 12.5% SDS-PAGE (large gel). M: position of prestained molecular weight markers indicated in kDa. *Lane 1:* strip of membrane probed with caspase 2/ICH-$1_{L/S}$ polyclonal antibody at 1:100 dilution, *lane 2*: strip of membrane incubated with caspase 2 monoclonal antibody at 1:1000 dilution.

The two anti-caspase 2 antibodies, both claimed to be caspase 2 specific, tested on the same Western blot detected two different protein bands in HeLa protein extracts with a molecular weight difference of approximately 3-4kDa. The Transduction

Laboratories monoclonal anti-caspase 2 antibody at 1:1000 dilution bound to a protein with an apparent molecular weight of approximately 45-46kDa, whereas the Santa Cruz anti-caspase 2 at 1:100 dilution bound to a larger protein of approximately 46-49kDa. A spokesperson from Transduction Laboratories indicated that a variety of unspecified further tests had been carried out and confirmed that the monoclonal anti-caspase 2 antibody was caspase 2 specific, whereas the polyclonal Santa Cruz Biotechnology antibody was subsequently temporarily withdrawn from the market. In subsequent immunoblot analysis of caspase 2 expression the commercial anti-caspase 2 monoclonal antibody supplied by Transduction Laboratories was thus used.

Raised against a large proportion of the caspase 2 protein (see Appendix 2, Figure 3.A) the antibody might in theory recognise either the large or the small subunit present in both pro- and active enzyme or it could bind to the linker region lost upon procaspase 2 activation. In order to determine whether the antibody recognised active caspase 2 large or small subunits, it was tested in Western blotting analysis for its ability to bind to any of the three recombinant caspase 2 fragments described in chapter 3.1 or the recombinant caspase 2 large and small subunits (provided by D.W. Nicholson from Merck Frosst) (data not shown). The commercial antibody failed to detect any of the three recombinant caspase 2 fragments and both the large and the small recombinant caspase 2. This surprising result might have two explanations. First, as the Transduction Laboratories antibody was raised against a recombinant polypeptide extending from amino acid 225-401, it is possible that the epitope detected lies in a region not included in any of the tested polypeptides. This would locate this epitope to the linker region between the large and the small subunit (amino acids 316-336). Alternatively, the Transduction Laboratories antibody might itself have an inappropriate specificity.

3.3.2. Caspase 2 expression in four human cell lines

Non-apoptotic whole cell extracts of four human cell lines, including Jurkat and H9 (human T leukaemia cell lines), HeLa (cervical carcinoma) and Priess (a DR4 homozygous EBV transformed B cell line), were prepared in order to analyse levels of caspase 2 expression by Western blotting (Figure 3.19.). Jurkat, HeLa and H9 cells were chosen because of their reported high expression of various caspases (see

chapter 1.6.1.), whereas human Priess B cells are of particular interest because of the potential role of caspase 2 in B cell apoptosis (Bergeron, unpublished data).

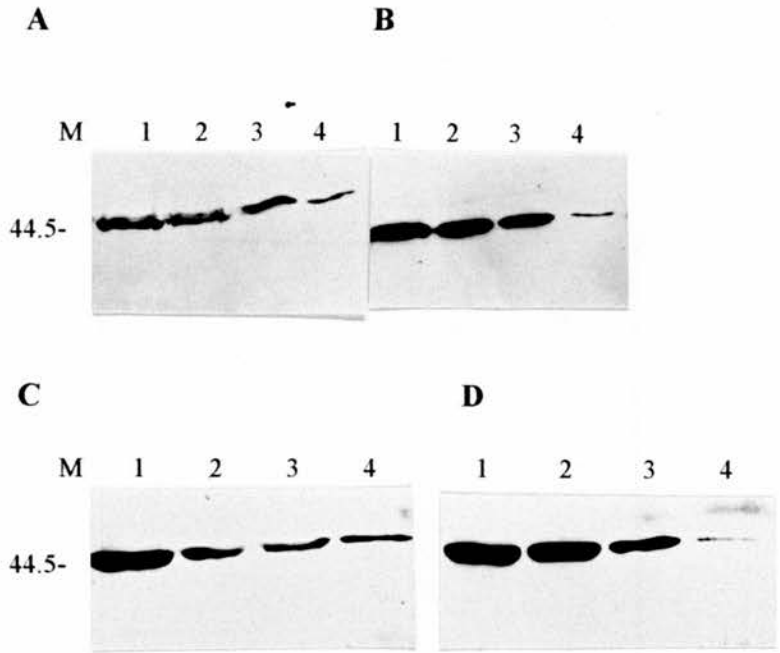

Figure 3.19. Immunoblot analysis of caspase 2 expression in four human cell lines. Whole cell protein extracts were prepared, quantitated, were subjected to SDS-PAGE (12.5% minigel) and blotted onto nitrocellulose membranes. All membranes were probed with anti-caspase 2 monoclonal antibody at 1:750 dilution. M: prestained molecular weight markers indicated in kDa. A-D). *Lane 1*: 15μg; *lane 2*: 10μg; *lane 3*: 5μg; *lane 4*: 1μg of whole cell protein extract was run. **A)** titration of Jurkat whole cell protein extract; **B)** titration of H9 whole cell protein extract; **C)** titration of HeLa whole cell protein extract; **D)** titration of Priess whole cell protein extract.

In all four cell lines a protein band of an apparent molecular weight of 46kDa was detected by the monoclonal anti-caspase 2 antibody, corresponding approximately to the predicted size of caspase 2 of 48kDa. High expression of the detected protein was found in all four cell lines. The monoclonal antibody appeared to have a high affinity for this protein, being able to detect it in as little as 1μg whole cell extract.

3.3.3. Immunocytochemical caspase 2 detection in three human cell lines

In order to analyse the subcellular localisation of caspase 2, cytospins of exponentially growing non-apoptotic Jurkat, HeLa and H9 were prepared and analysed by immunohistochemical staining (Figure 3.20.).

The anti-caspase 2 monoclonal antibody showed exclusively cytoplasmic staining in all three cell lines (Figure 3.20., panels A, C and E). Staining intensity varied depending on the cell density of the cytospin and was strongest in the H9 cytospin (Figure 3.20. panel E).

In summary, a commercially available monoclonal antibody raised against caspase 2 (Transduction Laboratories) was shown to bind strongly to a 45-46kDa protein in HeLa, Jurkat, H9 and Priess cell lines, and detected a cytoplasmic protein in immunocytochemical preparations of these cells. Although this suggested the monoclonal antibody could be a useful reagent, there remains some concern about its specificity: unlike the polyclonal antibodies described in section 3.2., it does not bind in Western blots to purified peptides covering all but 20 amino acid in recombinant caspase 2 of the monoclonal antibody immunogen. Moreover, the polyclonal antibodies and another commercial antibody to caspase 2 identify a protein in human cell extracts of perceptibly larger molecular weight (46-48kDa) in comparison with the Transduction Laboratories monoclonal antibody

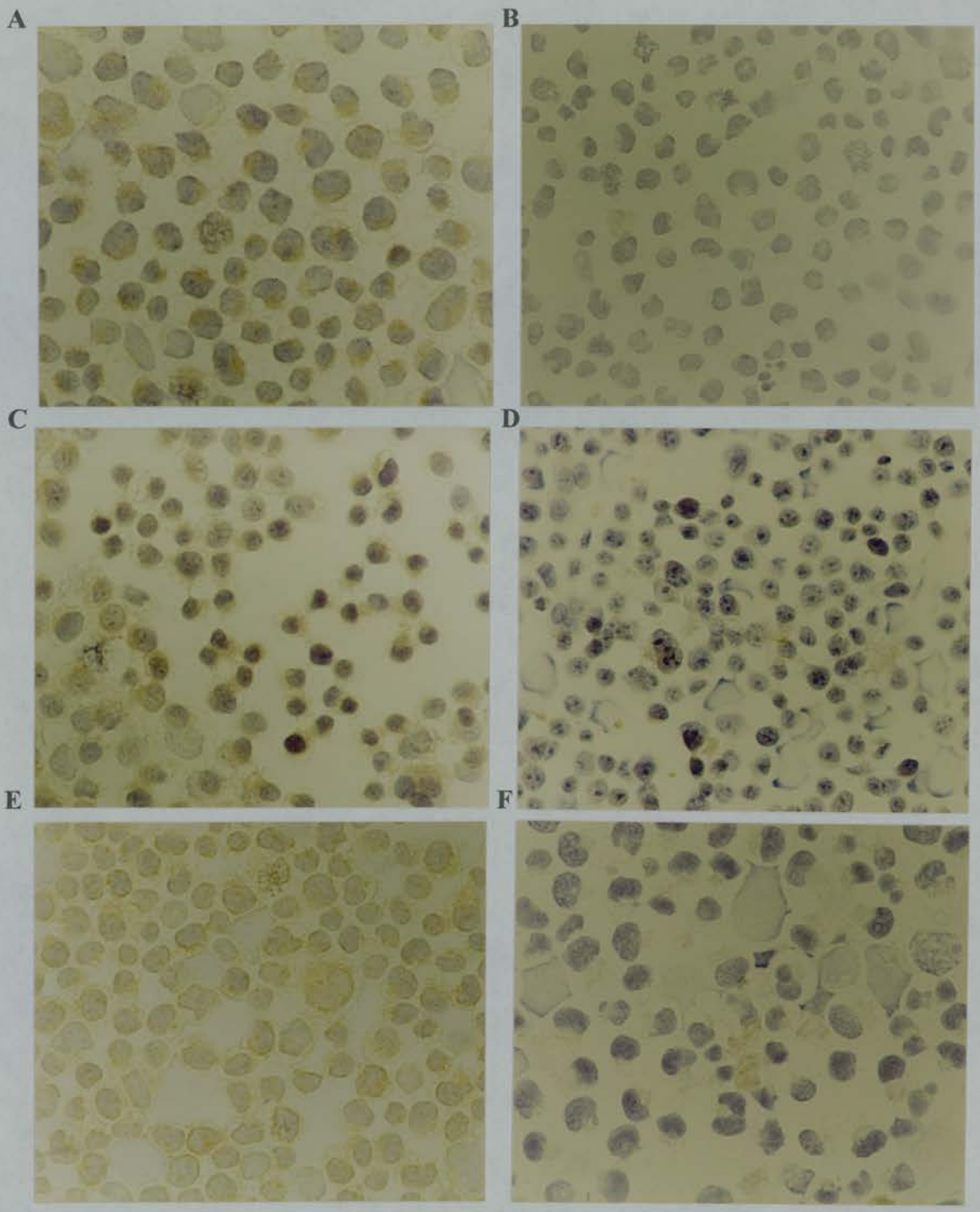

Figure 3.20. Caspase 2 immunocytochemical staining in three human cell lines. Cytospins incubated with anti-caspase 2 antibody at 1:30 dilution, secondary rabbit anti-mouse HRP coupled at 1:100 dilution, detection with DAB. Negative control slides were incubated with secondary antibody only. Phase contrast microscopy, magnification 400x. *Panel **A***: Jurkat cells incubated with anti-caspase 2 antibody; *panel **B***: Jurkat negative control cytospin; *panel **C***: HeLa cells incubated with anti-caspase 2 antibody; *panel **D***: Hela negative control; *panel **E***: H9 cells incubated with anti-caspase 2 antibody; *panel **F***: H9 negative control.

3.4. USE OF COMMERCIAL ANTIBODIES TO STUDY THE DISTRIBUTION OF CASPASE 2 IN HUMAN TISSUES

3.4.1. Analysis of caspase 2 expression in human tissue extracts

Despite the reservations over specificity, the strong reactivity of the Transduction Laboratories antibody in Western blotting and immunocytochemical preparations made it an attractive reagent with which to study caspase 2 distribution in healthy and diseased human tissues. Accordingly, the antibody the antibody was applied to Western blotting of extracts prepared from a variety of normal human tissues and to paraffin embedded sections of human tissues. Soluble protein extracts were prepared, including three different lung tissue extracts, and were analysed by Western blotting for levels of caspase 2 expression (Figure 3.20.). In parallel, all 13 tissues extracts were analysed in Coomassie stained SDS-PAGE gels to confirm equal protein loading and lack of obvious protein degradation (Figure 3.21.)

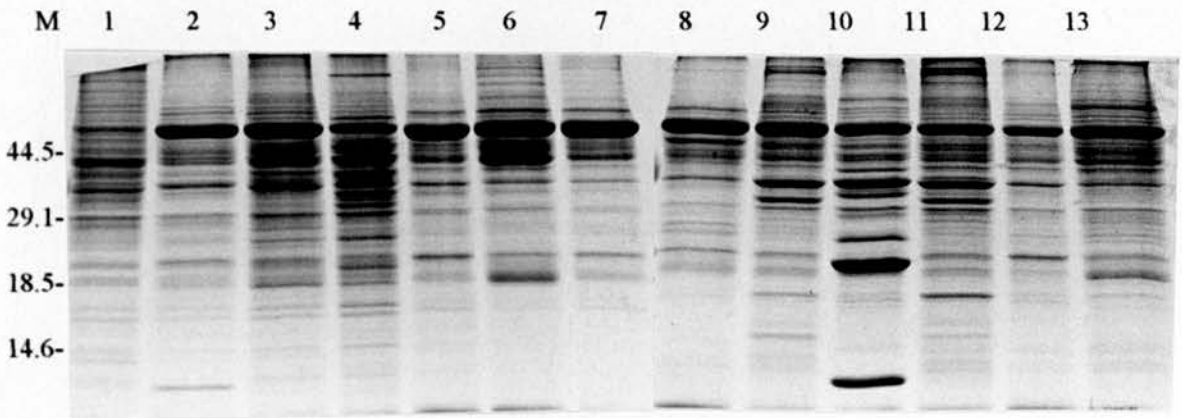

Figure 3.21. SDS-PAGE analysis of human tissue extracts. Soluble protein extracts of 13 human tissues were prepared, normalised for protein content (15μg/lane), subjected to SDS-PAGE (12.5% minigel), fixed and stained with Coomassie blue. M: molecular weight standards in kDa. *Lane 1*: cerebrum; *lane 2*: heart; *lane 3*: kidney; *lane 4*: liver; *lane 5*: lung (case 387); *lane 6*: lung (case 388); *lane 7*: lung (case 396); *lane 8*: pancreas; *lane 9*: prostate; *lane 10*: skeletal muscle; *lane 11*: small intestine; *lane 12*: spleen; *lane 13*: testes.

The Coomassie stained SDS-PAGE gel of the 13 human tissue protein extracts showed that equal amounts of total protein were loaded on the gel and samples were not subjected to proteolytic degradation.

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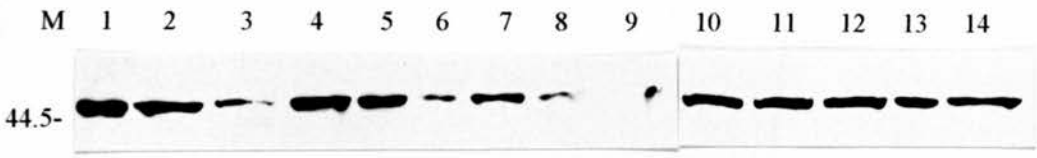

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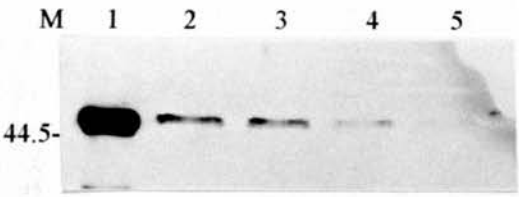

Figure 3.22. Immunoblot analysis of human tissues using the Transduction Laboratories monoclonal antibody to caspase 2. Soluble protein extracts were prepared from human tissues, normalised for protein content (15μg/lane) and subjected to SDS-PAGE (12.5% minigel), transferred to nitrocellulose and incubated with 1:750 dilution of anti-caspase 2 monoclonal antiserum. M: molecular weight markers in kDa. Jurkat whole cell extract (15μg) was run as a positive control on each blot. **A)** Immunoblot of 13 human tissue protein extracts. *Lane 1*: Jurkat extract; *lane 2*: cerebrum; *lane 3*: heart; *lane 4*: kidney; *lane 5*: liver; *lane 6*: lung (case 387); *lane 7*: lung (case 388); *lane 8:* lung (case 396); *lane 9*: pancreas; *lane 10*: prostate; *lane 11*: skeletal muscle; *lane 12*: small intestine; *lane 13*: spleen; *lane 14*: testes. **B)** Immunoblot of pancreatic protein extract at various concentrations. *Lane 1*: 15μg Jurkat extract; *lane 2*: 20μg; lane 3: 15μg; *lane 4*: 10μg; *lane 5*: 5μg pancreatic extract.

A protein with a molecular weight of around 46kDa was identified in all tissues analysed, although the relative levels varied between tissues (Figure 3.22.A). The highest levels were found in cerebrum, kidney, liver, lung (case 386), prostate, skeletal muscle, small intestine, spleen and testes, whereas expression was slightly lower in heart, pancreas and other lung cases. Titration of pancreatic tissue protein extract confirmed that the detected protein was present in the pancreas at slightly lower concentration (Figure 3.22.B).

3.4.2. Immunohistochemical analysis of normal human tissues

The Transduction Laboratories antibody was applied to human tissue sections from the same human source as the samples of the immunoblot analysis. Various antigen retrieval methods were explored (data not shown). Optimal staining results were obtained with microwaved sections; staining was little or absent using untreated or trypsinised sections. Figure 3.23. shows selected examples of the results achieved using the anti-caspase 2 monoclonal antibody and Table 3.4. summarises the results. Negative controls (secondary antibody only) produced negligible staining (data not shown).

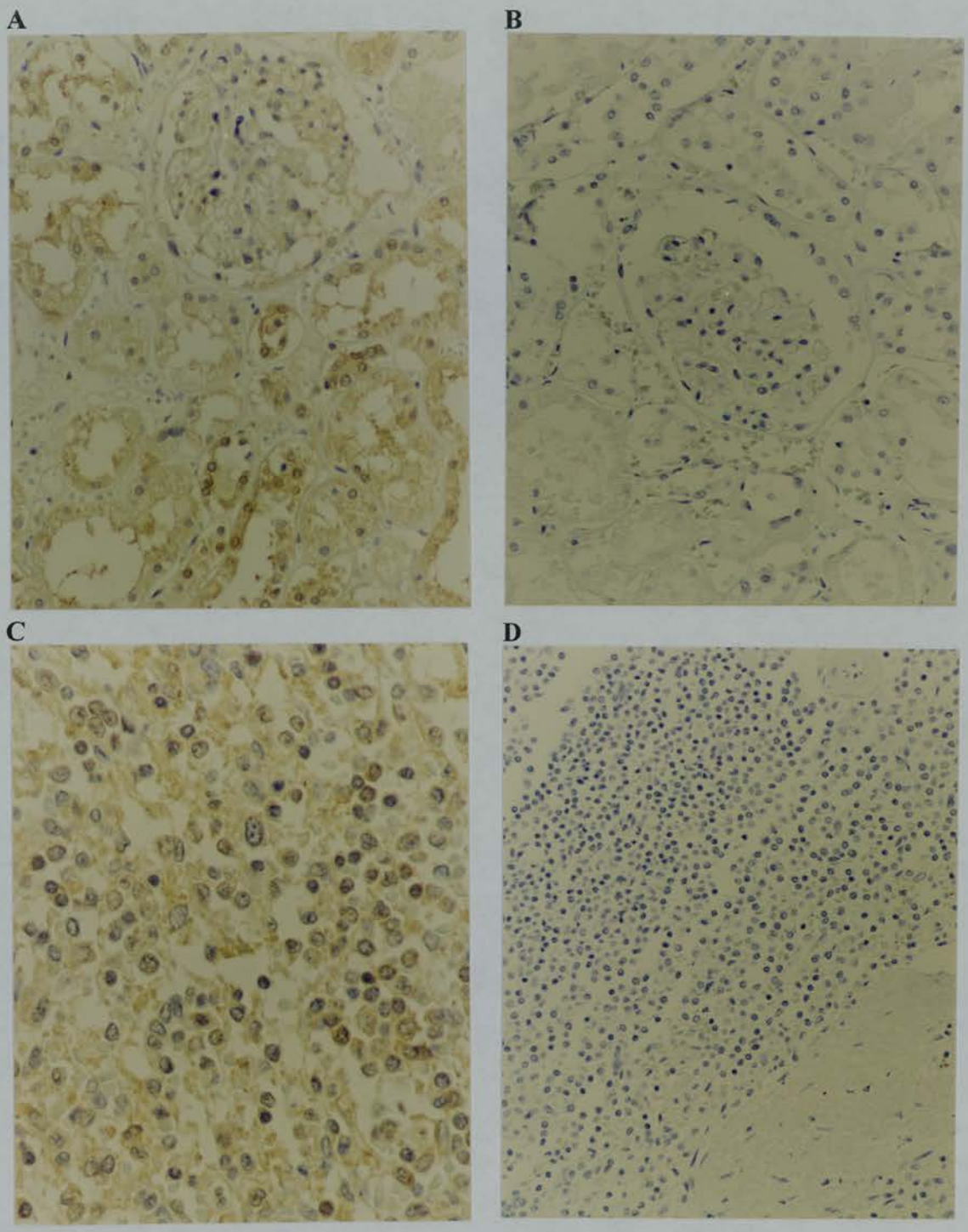

Figure 3.23. Representative examples of immunostaining in normal human tissue sections using the Transduction Laboratory antibody to caspase 2. Microwaved sections incubated with primary anti-caspase 2 monoclonal antibody at 1:30 dilution and secondary rabbit anti-mouse HRP-coupled antibody at 1:100 dilution, visualisation with DAB. Analysis by phase contrast microscopy, magnification indicated in brackets. *Panel A*: kidney anti-caspase 2 (200x); *panel B*: kidney negative control (200x); *panel C*: spleen anti-caspase 2 (400x); *panel D*: spleen negative control (200x).

Table 3.4. "Caspase 2" immunoreactivity in normal human tissues

Organ/Tissue	*Structure/Cell type*	*Caspase 2 intensity*
Pituitary gland		
Anterior pituitary gland		3
Colon	Epithelium	1
Heart		
Myocardium		0-1
Kidney		
Glomeruli		0
Collecting tubules	Proximal convoluted tubules	3
	Distal convoluted tubules	3
	Loop of Henle	3
	Collecting ducts	0
Lung		
Bronchial epithelium		1
Alveoli		0
Liver		
	Hepatocytes	1
	Bile duct epithelium	1
Lymph nodes/tonsil		
Germinal centre	Follicular dendritic cells	1
Mantle zone	Lymphocytes	1
Squamous epithelium		1
Nervous system		
Brain hemispheres and basal ganglia		0
Spinal cord		0
Pancreas		
Exocrine	Centri-Acinar ducts	1-2
	Ductal epithelium	1-2
Endocrine	Islets of Langerhans	2
Placenta	Chorionic villi	0
	Decidua	1
Prostate		
Glandular epithelium		1
Spleen	Marginal zone lymphocytes	2
Thymus	Hasselli Corpusdes thymocytes	0

The immunostaining was scored according to the intensity as 0 negative; 1 trace/weak; 2 definite; 3 intense.

Immunostaining was absent or weak in most tissues, but strong immunoreactivity was identified in selected cell types - notably the ductular and acinar cells of pancreas, renal tubular epithelium, and marginal zone lymphocytes in spleen.

3.4.3. Immunohistochemical analysis of, chronically inflamed and neoplastic human pancreas

Following the observations of immunoreactivity in normal pancreas, immunostaining with the Transduction Laboratory antibody was studied in chronic pancreatitis and pancreatic carcinoma in order to analyse whether the expression is modulated by the tissue disease status (Figure 3.24.). Results presented for normal, chronically inflamed and cancerous human pancreas was based on multiple immunohistochemical analysis (7 different cases each) of multiple immunostained slides. Table 3.5. summarises the results.

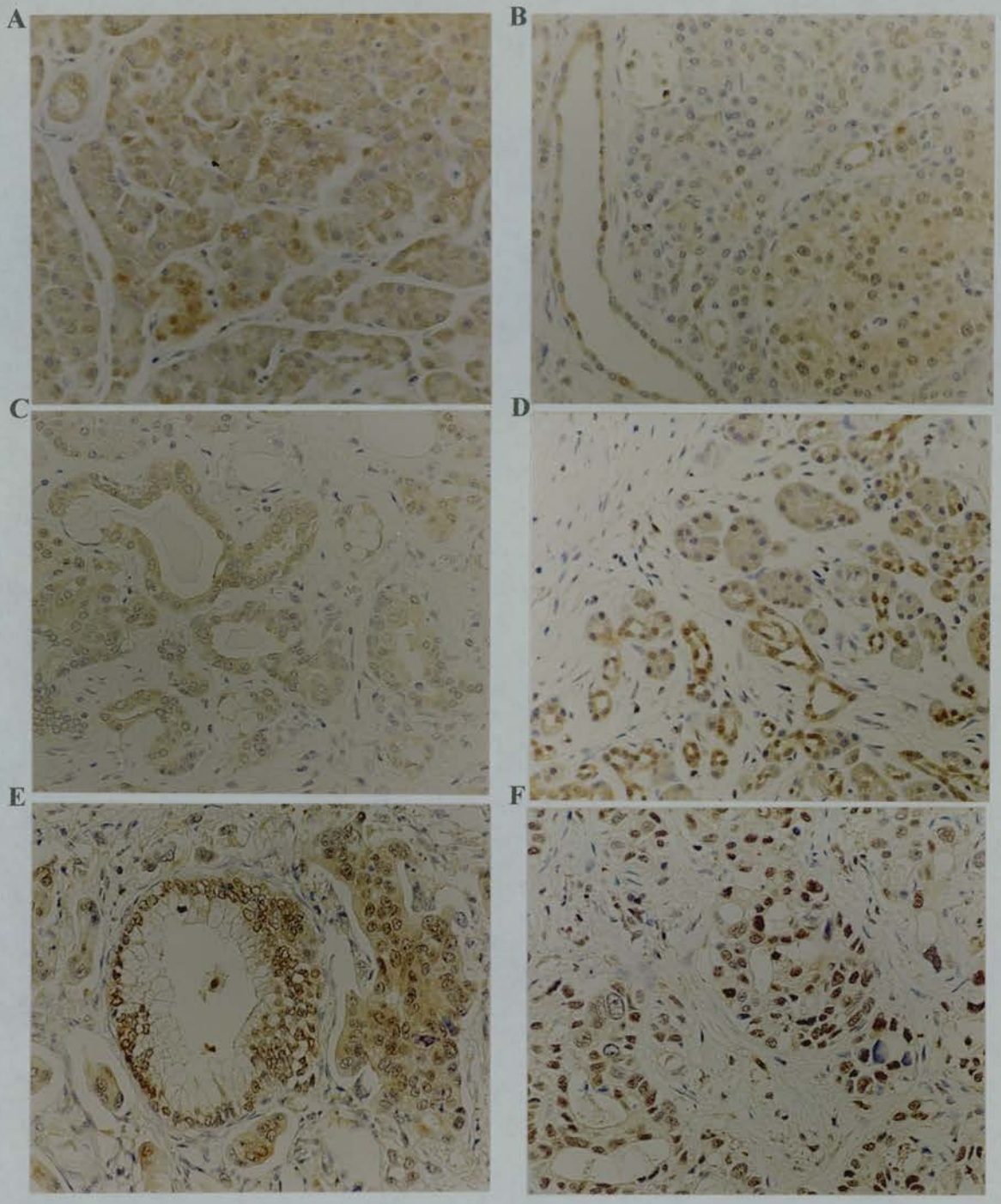

Figure 3.24. Representative examples of immunostaining in normal, inflamed and cancerous human pancreatic tissue sections using the Transduction Laboratory antibody to caspase 2. Microwaved sections incubated with primary anti-caspase 2 monoclonal antibody at 1:30 dilution and secondary rabbit anti-mouse HRP-coupled antibody at 1:100 dilution, visualisation by DAB; magnification 200x. For all tissues examined, the immunostaining procedure was performed in parallel. *Panels **A-B***: normal pancreas; *panels **C-D***: chronic pancreatis; *panel **E- F***: pancreatic cancer

Table 3.5. "Caspase 2" immunoreactivity in normal and diseased human pancreas.

Organ/Tissue	*Structure/Cell type*	*Caspase 2*
intensity		
Normal Pancreas		
Exocrine	Centriacinar ducts	1
	Ductal epithelium	1
Endocrine	Islets of Langerhans	2
Chronic Pancreatitis		
Exocrine	Centriacinar ducts	2
	Acinar ductal transformations	2
	Ductal epithelium	2-3
Endocrine	Islets of Langerhans	2
Pancreatic Cancer		
Exocrine	Acinar ducts	2
	Ductal epithelium	0-3

The immnostaining was arbitrarily scored according to the intensity as 0 negative; 1 trace/weak; 2 definite; 3 intense.

In normal pancreas the exocrine cells of the small ducts and centri-acinar ducts showed cytosolic staining, whereas the epithelial cells lining the pancreatic ducts were either unstained or contained only weak immunoreactivity. Both the α- and β-endocrine cells of the islets of Langerhans stained positive. Expression appeared enhanced in ductal epithelium in cases of chronic pancreatitis in comparison to normal and *de novo* expression was identified in areas of acinar-ductal transformation. In ductal carcinoma there was widespread but heterogenous expression in malignant epithelium. Particularly intense staining was evident in malignant glands in pseudopapillary projections into the gland lumen. In the malignant tumours, apoptotic cells were identified, and they were either unstained or weakly immunoreactive.

In summary, the Transduction anti-caspase 2 monoclonal antibody clearly detected a 46kDa protein in Western blotting, immunocytochemistry and immunohistochemistry, with an apparent tissue specific staining pattern, whereas the anti-fragment 1 and 2 antibodies only very weakly bound to a protein of the predicted molecular weight of caspase 2 in Western blotting analysis of human tissues extracts and were nonfunctional in immunohistochemistry. Nonetheless the Transduction Laboratories monoclonal antibody leaves some confusion about its specificity as the antibody failed to detect any of the recombinant fragments and recombinant caspase 2 subunits and bound to a protein of approximately 46kDa, varying from the predicted molecular weight of 48kDa for procaspase 2.

3.5. INDUCTION OF APOPTOSIS IN HUMAN CELL LINES

Hitherto in this thesis, the emphasis has been to establish reagents specific for caspase 2, in order to study the presumptive role of this enzyme in apoptosis. In the experiments to follow the aim is rather to initiate *bona fide* apoptosis in cell systems of interest and observe the impact of this process on the proteins detected by these reagents. Two lymphoid cell lines are studied, Jurkat T cells and Priess B cells. Jurkat cells were chosen because the initiation of apoptosis in these cells has been particularly well-studied by other laboratories and they are known to express the majority of caspases. Priess B cells were chosen because of the suggested role of caspase 2 in B cell apoptosis from caspase 2 knock-out studies (see chapter 1.6.3.3). Three very different stimuli were applied arising in the cytoplasm (staurosporine), nucleus (etoposide) and the cell surface (Fas). Apoptosis was assessed by morphological analysis and by immunoblotting analysis of cleavage of PARP, a well characterised caspase substrate cleaved during apoptosis. Involvement of caspase 2 and 3 was sought from evidence of their proteolysis during apoptosis.

3.5.1. Detection of apoptosis in Jurkat and Priess cells

To establish the time course of development of apoptosis in Jurkat and Priess cells, the cells were harvested, following the application of the lethal stimulus, for the study of apoptotic morphology (Figure 3.25., data shown for Fas-induced cells only). Morphological confirmation and quantification of apoptosis was assessed by studying cells, stained with acridine orange, under a fluorescence microscope. Cells with condensed or fragmented nuclei surrounded by cell membrane, exhibiting bright fluorescent green nuclear staining were scored as apoptotic. Cells showing shrinkage but no chromatin condensation, therefore showing a more diffuse fluorescent green nuclear staining, were scored as normal. Jurkat and Priess cells treated with each of the apoptotic inducing agents showed the same characteristic changes of apoptosis. Jurkat and Priess cells with normal cell morphology showed a characteristic opaque green staining of the nucleus (Figure 3.25., panels A and B), early apoptotic cells showed cell shrinkage with nuclear condensation visible as fluorescing bright green nuclei and late apoptotic cells were further condensed in cell size and showed the formation of multiple nuclear fragments.

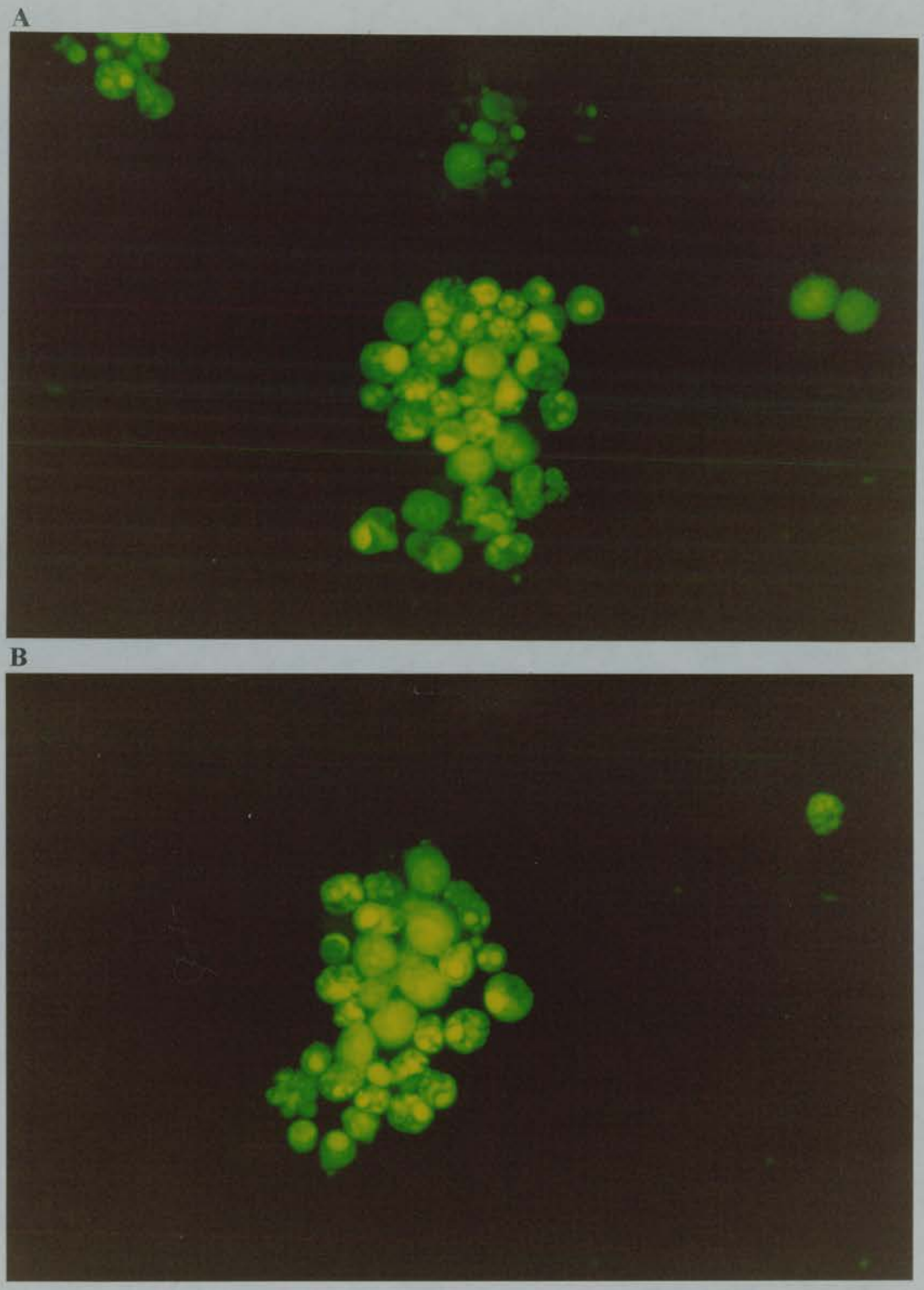

Figure 3.24. Fluorescence microscopic analysis of Fas-induced Jurkat and Priess cells. Cells induced with 200ng/10^6cell anti-Fas antibody. Photographs of cells were taken at a 6 hour time point on a fluorescence microscope at 400x magnification. *Panel A*: Jurkat cells; *panel B*: Priess cells.

3.5.2. Concentration dependent induction of Jurkat cell apoptosis by staurosporine

In order to determine the optimal concentration of staurosporine sufficient to induce apoptosis in Jurkat cells, Jurkat cells were treated with concentrations of staurosporine up to 5μM and apoptosis was assessed by fluorescence microscopy over a 22 hour time course. Figure 3.26. shows the effect of different staurosporine concentrations on the rate of Jurkat cell apoptosis.

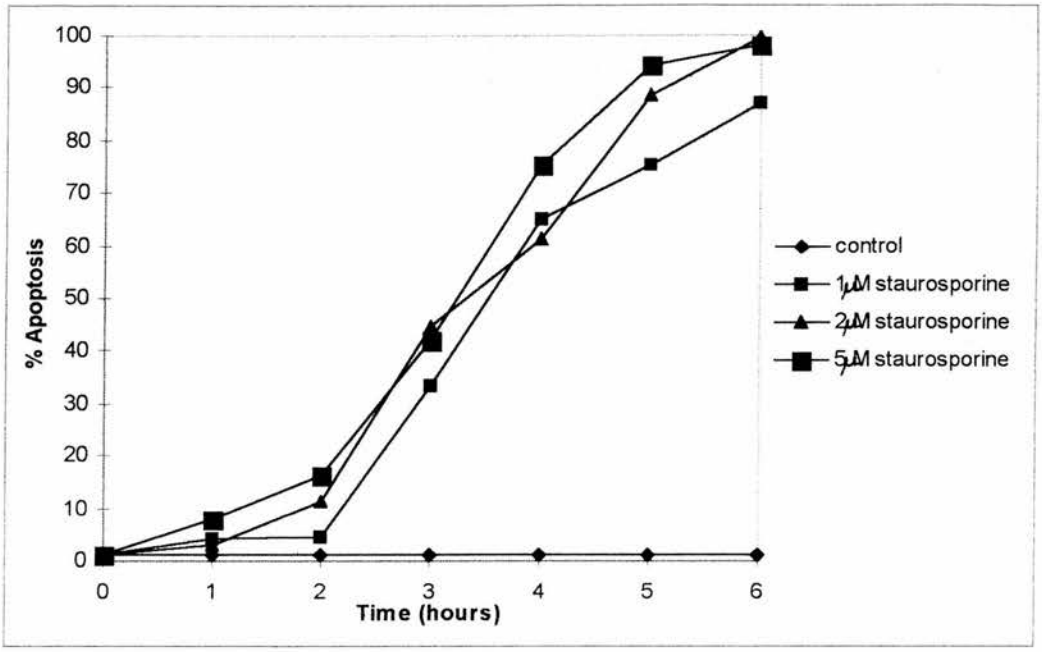

Figure 3.25. Concentration dependent induction of apoptosis by staurosporine. Jurkat cells were incubated with 1μM, 2μM and 5μM staurosporine and were analysed at the indicated time points for apoptotic morphology, controls received no treatment.

At all three concentrations staurosporine potently induced apoptosis in Jurkat cells resulting in about 85-95% apoptotic cells at 6 hours. At a concentration of 1μM, however, a later and more gradual onset of apoptosis was observed with less apoptotic cells at 2 hours. In order to analyse early apoptotic events with only a small percentage of the cell population undergoing apoptosis and to be able to compare those with later time points with mainly apoptotic cell population 1μM concentration of staurosporine was chosen for future experiments.

3.5.3. Time course of induction of apoptosis in Jurkat and Priess cells with three different stimuli

Jurkat and Priess cells were induced to undergo apoptosis with etoposide, anti-Fas antibody and staurosporine. At each time point protein extracts for immunoblotting were prepared and cell extracts were analysed under a fluorescence microscope for apoptotic morphology (Figure 3.27.).

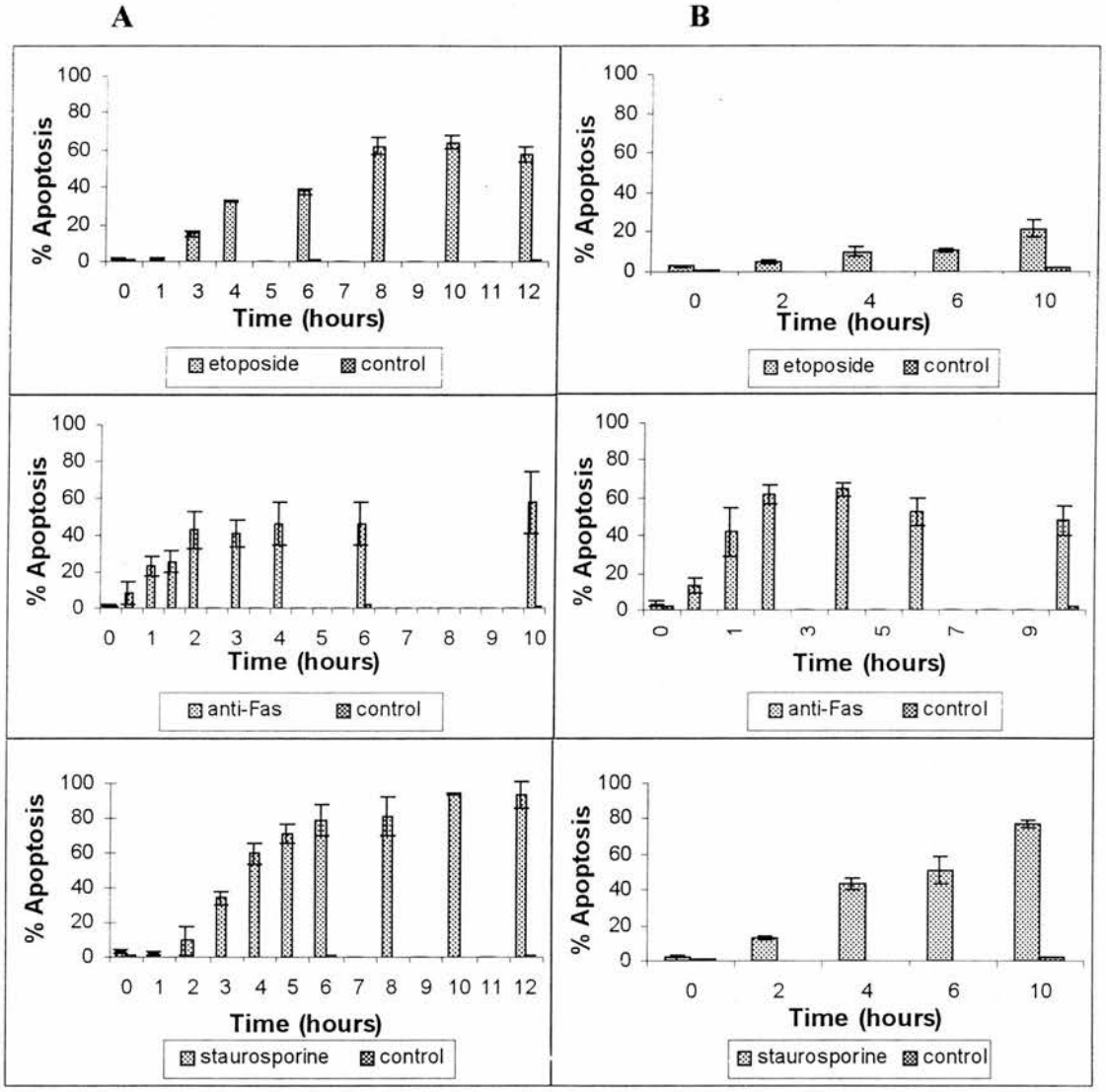

Figure 3.27. Time course of apoptosis in Jurkat and Priess cells following incubation with etoposide, anti-Fas antibody and staurosporine. Jurkat and Priess cells were incubated for up to 12 hours either in medium alone (control) or in the presence of 50μM etoposide, 200ng/10^6 cells anti-Fas antibody or 1μM staurosporine (induced). At indicated time points after the addition of the stimulus aliquots of cell were taken and scored for apoptotic morphology. **A)** Jurkat cells; **B)** Priess cells.

All three agents induced apoptosis in both cell lines, although with different kinetics and overall efficiency. Anti-Fas treatment resulted in the rapid induction of apoptosis (20% apoptotic cells at 1 hour), whereas etoposide and staurosporine treatment only reached similar values between two and four hours. At the given concentration staurosporine was the most effective inducing agent rendering about 90% cells apoptotic at 12 hours in both cell lines, whereas etoposide treatment was much less effective in inducing apoptosis, particularly, in the case of Priess cells. This indicated that Priess cells might be more resistant to DNA damage and might require a higher etoposide dose to reach similar apoptotic values. Control cells for both lines showed less than 2% apoptosis throughout the study period of 12 hours. All three agents thus proved to be suitable to study caspase 2 and 3 activation in potentially different apoptotic signalling pathways.

3.5.4. Analysis of apoptotic cell morphology by electron microscopy

In order to analyse the detailed apoptotic morphology of apoptotic Jurkat and Priess cells and to compare morphological changes induced by different apoptotic stimuli, samples of apoptotic cells were prepared for electron microscopy and analysed in a transmission electron microscope (Figure 3.28.).

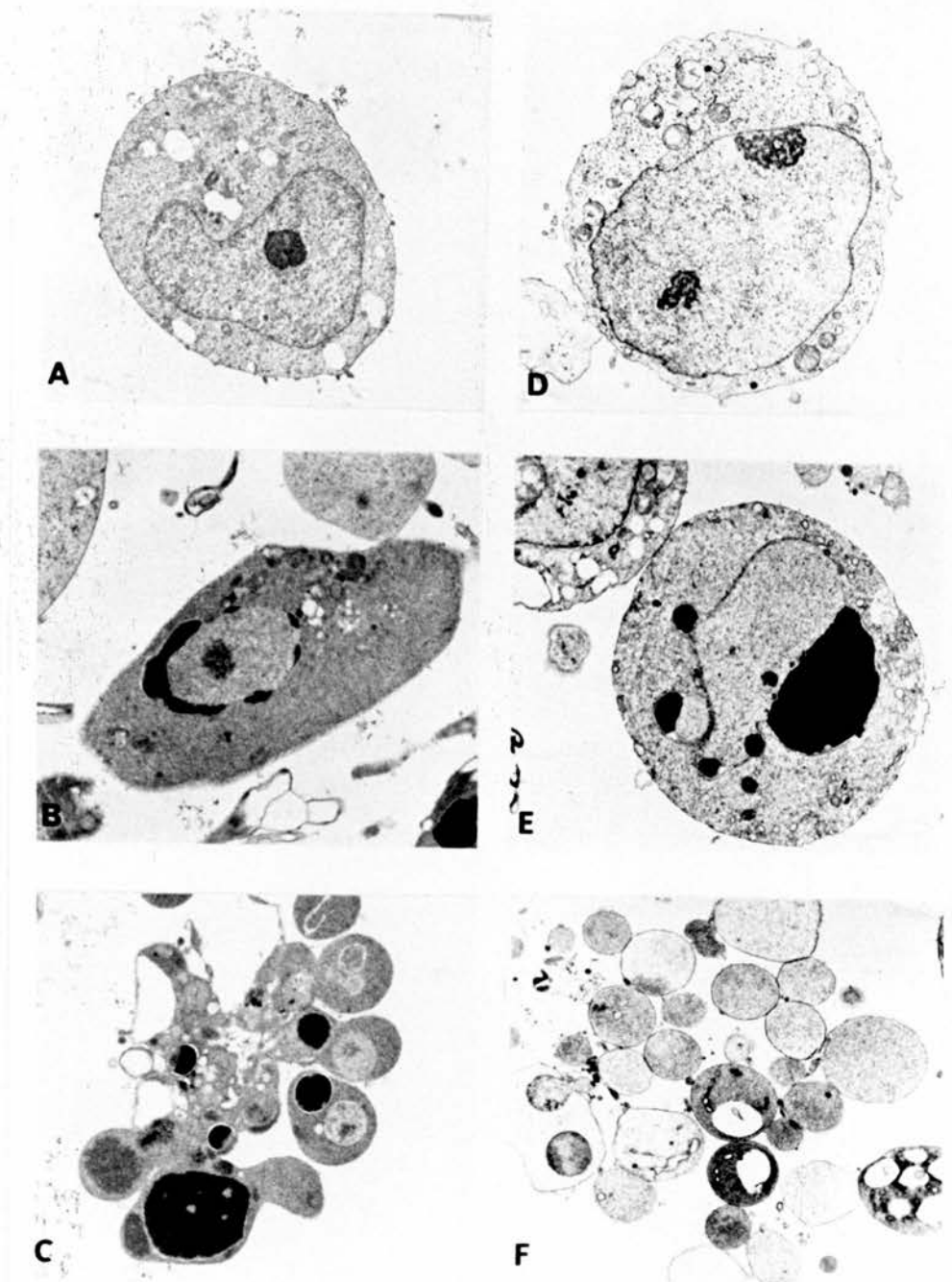

Figure 3.28. Electron microscopic analysis of Jurkat and Priess cells. Cells induced with 200ng/10^6cell anti-Fas antibody, 1μM staurosporine or 50μM etoposide. *Panels A, B* and *C*: apoptotic Jurkat cells, *panels D, E* and *F*: apoptotic Priess cells.

Detailed morphological analysis by electron microscopy of Jurkat and Priess cells treated with each of the reagents showed the characteristic structural changes of apoptosis (Figure 3.28., panels A-F). In particular the nuclear changes appeared identical regardless of the lethal stimulus, including chromatin condensation, usually in a circumferential orientation. Nuclear pore complexes were degraded only adjacent to decondensed chromatin and nuclear splitting to multiple fragments was observed with all stimuli. Cytoplasmic shrinkage with dilation of the endoplasmic reticulum was also observed with all stimuli. Mitochondrial morphology in early apoptosis was normal. Occasional cells, however, showed changes more akin to necrosis, with cytoplasmic changes including degenerate organelles and a distorted nucleus without the extensive condensation of apoptosis.

3.6.6. Analysis of PARP cleavage in apoptotic Jurkat and Priess cell extracts

Cleavage of caspase 3, caspase 2 and PARP, the classical caspase 3 substrate were studied by Western blotting of extracts harvested from Jurkat and Priess cells, following application of the three lethal stimuli in phase with the morphological changes. Western blots were probed with a human PARP antibody, which recognises the 116kDa enzyme and the larger fragment (89kDa) cleaved from it in apoptosis (Figure 2.29.).

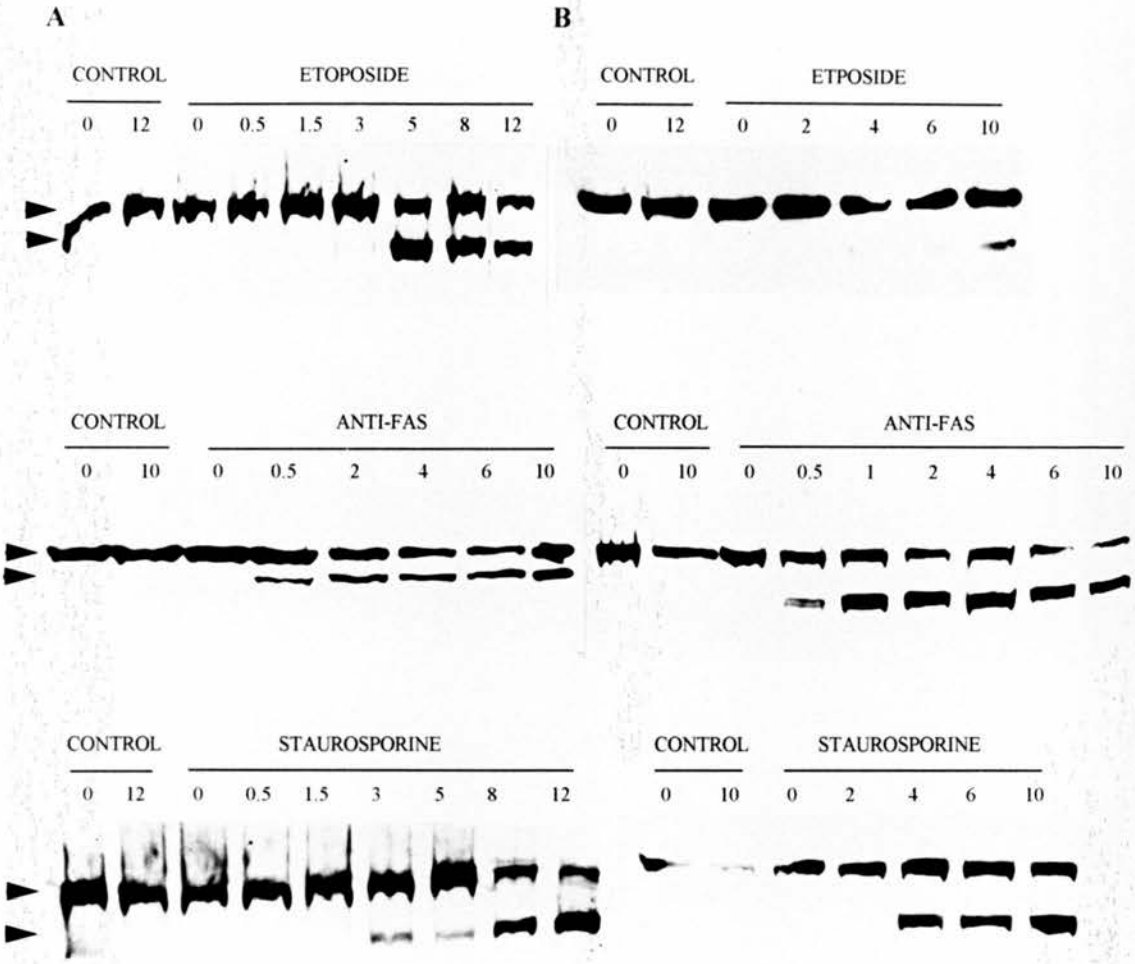

Figure 3.29. Immunoblot analysis of PARP cleavage in etoposide, anti-Fas and staurosporine treated Jurkat and Priess cells. Both human cell lines were treated continuously with either 50μM etposide, 200ng anti-Fas antibody or 1μM staurosporine and whole cell extracts were prepared at the indicated time points (hours). 15μg/lane was subjected to SDS-PAGE (7.5% minigel), transferred to nitrocellulose and incubated with human anti-PARP monoclonal antibody at 1:750 dilution. The position of the full length PARP (116kDa) and the cleaved large fragment (89kDa) is indicated by arrows.. *Panel A:* Jurkat whole cell lysate; *panel B*: Priess cell whole cell lysate.

PARP was cleaved in both cell lines during apoptosis induced with all three stimuli. This cleavage correlated closely with the kinetics of appearance of morphology of apoptosis for all three stimuli (see Figure 2.27.). PARP cleavage was first visible in both cell lines, at the 0.5h hour time point in Fas-induced apoptosis, corresponding to approximately 10% morphologically assessed apoptotic cells in culture. PARP cleavage was longest delayed in etoposide-induced apoptosis, in particular of the Priess cells, where a faint band corresponding to the cleaved product became, apparent at 4 hours, again at about 10% apoptosis. This became clearly visible only after 10 hour treatment corresponding, to approximately 25% apoptotic cells. In staurosporine-induced apoptosis, PARP cleavage was visible after 3 hour (Jurkat) and 4 hour (Priess) treatment, when approximately 40% of the cells were already undergoing apoptosis. At no time point following induction with any of the three stimuli was PARP cleaved to completion corresponding with the fact that throughout the entire studied treatment period both cell lines retained some non-apoptotic cells.

3.5.7. Analysis of caspase 3 status in apoptotic Jurkat and Priess cell extracts

The kinetics of caspase 3 activation were examined by analysis of procaspase 3 in immunoblots of extracts from Jurkat and Priess cells during the induction of apoptosis (Figure 3.30.).

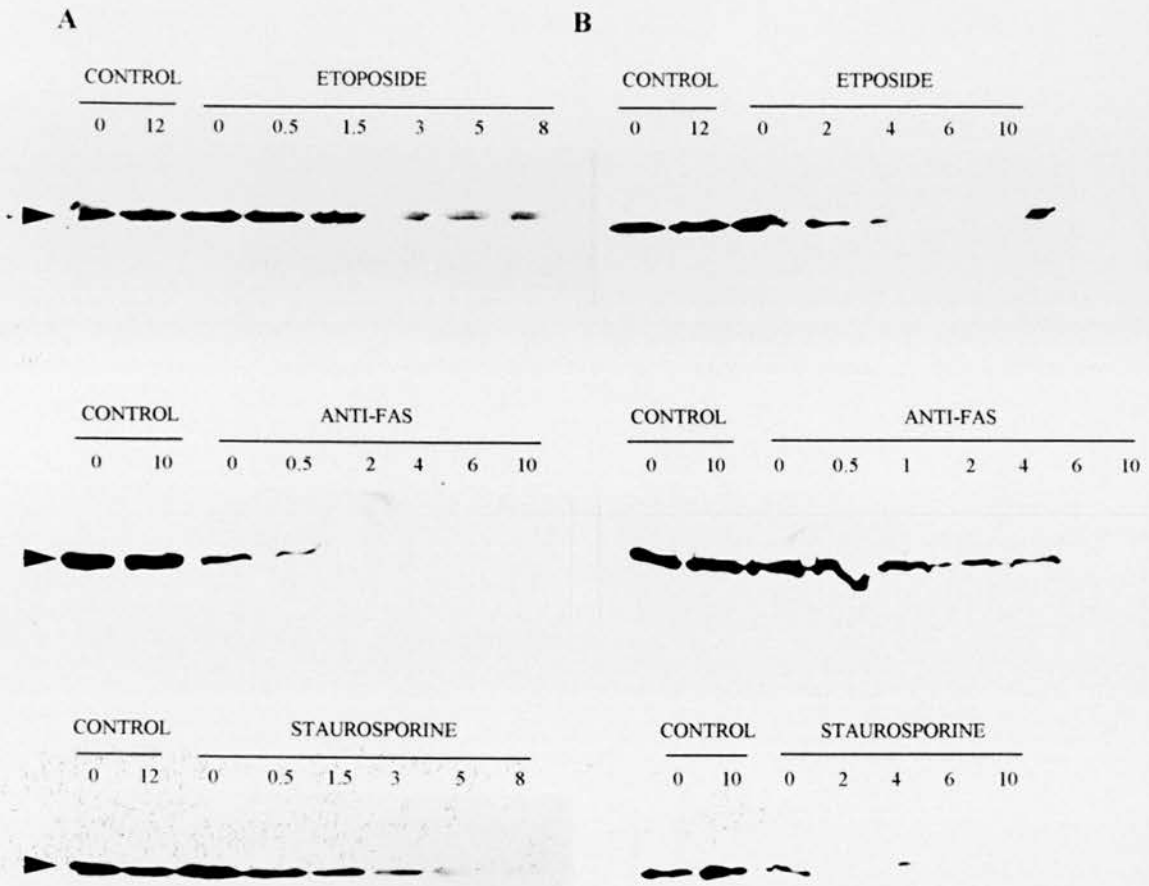

Figure 3.30. Immunoblot analysis of procaspase 3 in etoposide, anti-Fas and staurosporine treated Jurkat and Priess cells. Both human cell lines were treated continuously with 50μM etposide, 200ng anti-Fas antibody or 1μM staurosporine and whole cell extracts were prepared at the indicated time points. 15μg/lane whole cell extract was subjected to SDS-PAGE (12.5% minigel), transferred to nitrocellulose and incubated with human anti-caspase 3/CPP32 monoclonal antibody at 1:750 dilution. The position of the proenzyme form of caspase 3 (32kDa) is indicated by an arrow. The time of preparation of lysates is indicated at the top of each panel. *Panel **A:*** Jurkat whole cell lysate, *panel **B:*** Priess cell whole cell lysate.

In control cell lysates of both cell lines the anti-caspase 3/CPP32 monoclonal antibody reacted with a single band of approximately 32kDa corresponding to the predicted molecular weight of procaspase 3. During induction of apoptosis in both cell lines and with all three stimuli procaspase 3 levels decreased to low or undetectable amounts: at 30 min in Fas induced apoptosis, at 3 hours in staurosporine treated cells and 5 hours in etoposide treated cells. For each stimulus and in both cell lines this marked decrease in procaspase 3 level correlated closely with the percentage of total apoptosis induced by each stimulus (see Figure 3.27.) and the occurrence of the PARP cleavage (see Figure 3.29.). The results therefore suggest activation of caspase 3 by cleavage in both cell types by all three stimuli. Lower molecular weight fragments, corresponding to expected cleaved caspase 3 subunits of 17kDa and 12kDa, were not identified, even after prolonged exposure of the blots. Failure to detect this cleavage product may result from differential stability of proenzyme and cleaved subunits within the apoptotic cell. It should be also noted, however, that many low molecular weight proteins are poorly retained on nitrocellulose filters (due to a faster transfer than higher molecular weight proteins), which could contribute to the inability to detect the 17kDa and 12kDa caspase 3 subunits by immunoblotting and this also could contribute to the difficulty in their detection. However, it is also possible that the epitope detected by this monoclonal antibody lies within the prodomain: Transduction Laboratories are unable to provide any information regarding the antibody epitope (see Appendix 2, Figure 4. Antigen for caspase 3 monoclonal antibody).

3.5.8. Analysis of caspase 2 status in apoptotic Jurkat and Priess cell extracts

The previous results have demonstrated evolution of apoptosis in terms of caspase 3 activation and caspase substrate cleavage. To assess the status of caspase 2, and at the same time examine the credentials of several antibodies raised against it, the same extracts were therefore used in Western blotting analysis with the Transduction Laboratories anti-caspase 2 monoclonal antibody (Figure 3.31), two further polyclonal anti-caspase 2 antibodies obtained from the laboratories of J. Yuan and D.N. Nicholson (Figures 3.32. and 3.33.) and the two polyclonal antibodies (to

caspase 2 fragment 1 and 2) described in sections 3.1. and 3.2. of this thesis (Figures 3.34. and 3.35.).

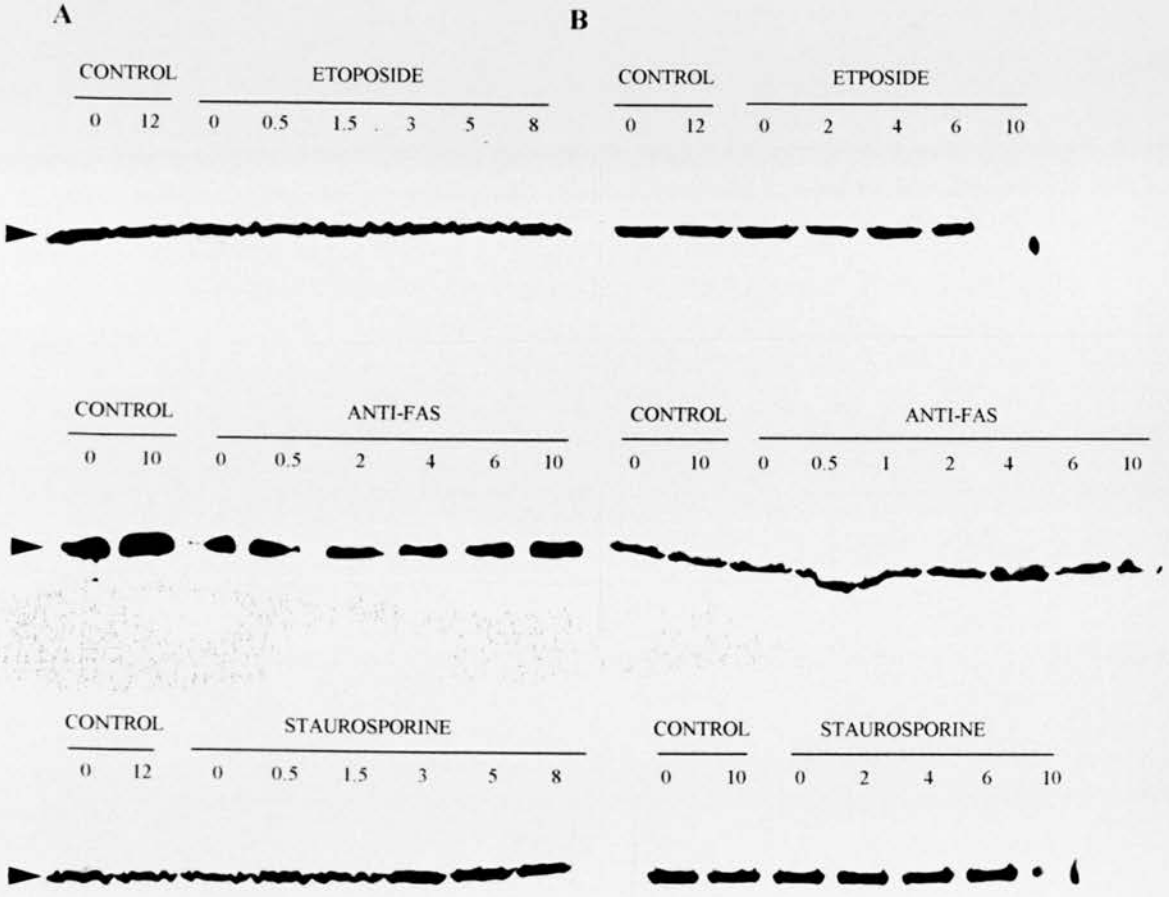

Figure 3.31. Immunoblot analysis of procaspase 2 in etoposide, anti-Fas and staurosporine treated Jurkat and Priess cells (monoclonal anti-caspase 2 antibody). Both human cell lines were treated continuously with either 50μM etoposide, 200ng anti-Fas antibody or 1μM staurosporine and whole cell extracts were prepared at the indicated time points. 15μg/lane whole cell extract was subjected to SDS-PAGE (12.5% minigel), transferred to nitrocellulose and incubated with human anti-caspase 2 monoclonal antibody at 1:750 dilution. The position of the proenzyme form of caspase 2 is indicated by an arrow. The time of preparation of lysates is indicated at the top of each panel. *Panel **A***: Jurkat whole cell lysate, *panel **B***: Priess cell whole cell lysate.

With all three stimuli and in both cell lines the 46kDa protein detected with the Transduction Laboratories anti-caspase 2 monoclonal antibody remained unchanged in quantity or molecular weight during the time course of apoptosis. No consumption of the detected protein band occurred during apoptosis with any of the three stimuli under conditions in which caspase 3 was clearly activated, PARP cleavage occurred and the molecular changes of apoptosis were evident (see Figures 3.27., 3.29. and 3.30.).

In contrast when the same cell lysates were analysed by immunoblotting for levels of procaspase 2 and caspase 2 subunits with two polyclonal anti-caspase 2 antibodies supplied by J. Juan and D.W. Nicholson (Figure 3.32., results shown for Fas induced apoptosis only) substantially different results were obtained which were further confirmed by new data obtained with the original 68AB/fragment 1 (prodomain) and BX08/fragment 2 (small subunit) anti-caspase 2 polyclonal antibodies (Figures 3.33. and 3.34.).

The polyclonal anti-caspase 2 antibodies provided by J.Yuan and D.W. Nicholson have been shown to bind to the full length procaspase 2, the large p18 subunit and 32-33kDa cleavage intermediate which either consists of the prodomain plus the large p18 subunit or the large subunit together with the small p12 subunit and the linker (Nicholson, personal communication; Li *et al*, 1997).

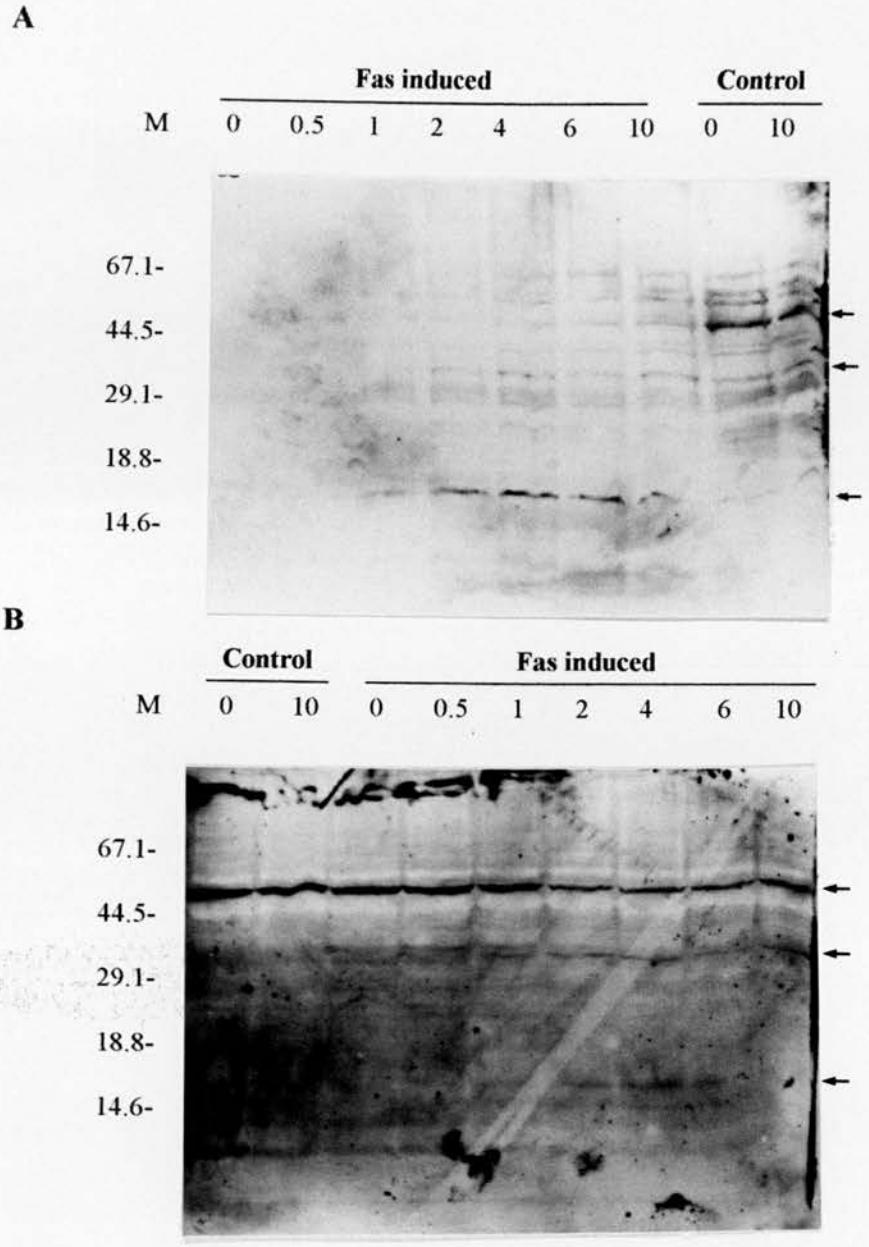

Figure 3.32. Immunoblot analysis of caspase 2 levels in anti-Fas treated Priess cells with two polyclonal anti-caspase 2 antibodies. Priess cells were induced with 200ng anti-Fas antibody or 1μM staurosporine, whole cell extracts were prepared at the indicated time points. 15μg/lane whole cell extract was subjected to SDS-PAGE (12.5% minigel), transferred to nitrocellulose and incubated with **A)** human anti-caspase 2 polyclonal antibody at 1:2000 dilution (D.W. Nicholson) and **B)** human anti-caspase 2 polyclonal antibody at 1:3000 dilution (J. Yuan). The positions of the proenzyme form of caspase 2 (48kDa) and activated enzyme are indicated by arrows. The time of preparation of lysates is indicated at the top of each panel. M: prestained molecular weight standards indicated in kDa.

Both polyclonal anti-caspase 2 antibodies proved to have a very low affinity towards caspase 2 and the recommended protocols reflected this. In Fas induced Priess B cells both antibodies were able to detect a protein band of approximately 48kDa corresponding to the predicted molecular weight of procaspase 2. During the ten hour time course this band decreased in intensity correlating with the appearance of two lower molecular weight bands of approximately 32kDa and 17kDa, which approximately correspond to caspase 2 large subunit (18kDa) and to a caspase 2 cleavage intermediate of 32kDa. These potential caspase 2 subunits were detected by the anti-caspase polyclonal antibody (D.W. Nicholson) at the 1 hour time point (Figure 3.32.A) at which the level of procaspase 2 band was reduced. These findings were confirmed in immunoblot analysis with the other anti-caspase 2 polyclonal antibody (J. Yuan) (Figure 3.32.B). A slight decrease of procaspase level is detectable from the 2 hour time point onwards, with potential caspase 2 cleavage intermediate and large subunit weakly detected in the 1 hour Fas extract and a stronger band visible in the 2, 4 and 6 hour extracts.

Western blotting analysis of staurosporine induced Jurkat and Priess cell extracts with both non-commercial anti-caspase 2 antibodies also suggests caspase 2 activation and proenzyme consumption (data not shown). Binding of both antibodies to procaspase 2 was much reduced in Jurkat and Priess whole cell protein extracts, which had previously been thawed and refrozen (2-4 times) and no binding to the active enzyme subunits was detectable in those extracts, pointing to the instability of the caspase 2 subunits.

These two polyclonal antibodies were subsequently tested by Western blotting analysis for their ability to detect any of the three recombinant caspase 2 fragments and the caspase 2 subunits provided by D.W. Nicholson (data not shown). Both anti-caspase 2 polyclonal antibodies bound to the large (p18) caspase 2 subunit but failed to recognise the prodomain or the small subunit. On the basis that both antibodies do not bind to the small subunit of caspase the 32-33kDa intermediate processing product of caspase 2 was believed to be the prodomain plus the large subunit, confirming previously published procaspase 2 processing (Li *et al*, 1997).

To further examine whether caspase 2 is activated in Jurkat cells induced to undergo apoptosis with etoposide (50μM) and staurosporine (1μM), whole cell lysates were prepared over an eight hour time course and subjected to Western blotting analysis using the two polyclonal antibodies raised against fragment 1/prodomain (68AB) (Figure 3.34.) and fragment 2/small subunit (BX08) (Figure 3.35.).

In this experiment Jurkat cells induced with staurosporine were approximately 85% apoptotic at the 8 hour time point, a figure closely similar to that obtained earlier (see Figure 3.31), while the Jurkat cells induced with etoposide were only 55% apoptotic at the 8 hour time point, showing about 25% less apoptosis than a previously established 8 hour average in Jurkat cells (see Figure 3.31.). Control cells in both experiments showed less than 2% apoptosis. Whole cell extracts from both experiments were analysed for caspase 3 cleavage (Figure 3.33.) In staurosporine treated Jurkat cells a reduction in procaspase 3 levels corresponding to caspase 3 activation was visible at the 4 hour time point, was further reduced at 6 hours and nondetectable at the 8 hour time point (Figure 3.33.A). In Jurkat cells treated with etoposide a slight reduction in procaspase 3 levels was apparent at 8 hours corresponding to approximately 55% apoptosis (Figure 3.33.B).

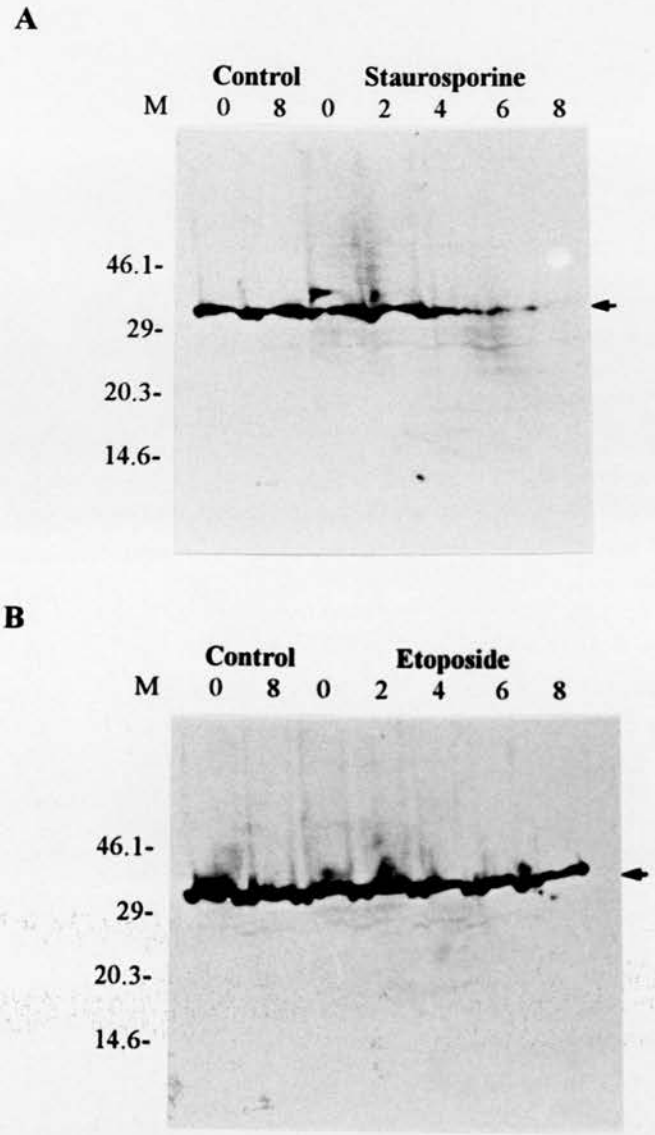

Figure 3.33. Immunoblot analysis of caspase 3 activation in staurosporine and etoposide treated Jurkat cells. Jurkat cells were induced with 1μM staurosporine (**A**) or 50μM etoposide (**B**), whole cell extracts were prepared at the indicated time points. 30μg/lane whole cell extract was subjected to SDS-PAGE (12.5% minigel), transferred to nitrocellulose and incubated with anti-caspase 3 monoclonal antibody at 1:750 dilution (Transduction Laboratories) The positions of the proenzyme form of caspase 3 (32kDa) indicated by an arrow. The time of preparation (in hours) of lysates is indicated at the top of each panel. M: prestained molecular weight standards indicated in kDa.

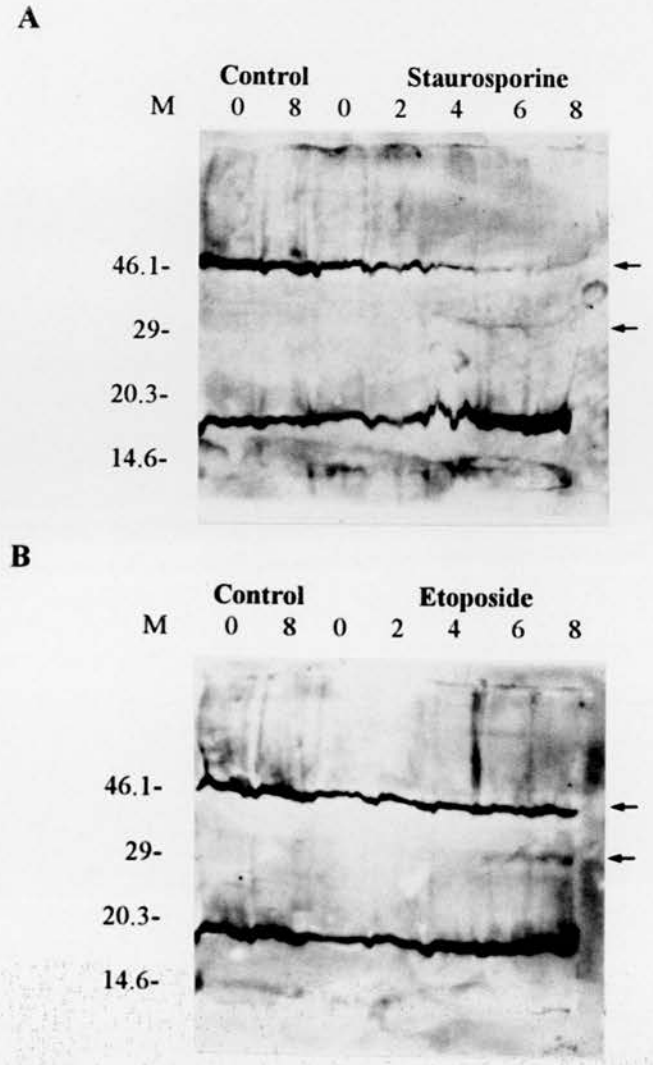

Figure 3.34. Immunoblot analysis of caspase 2 levels in staurosporine and etoposide treated Jurkat cells. Jurkat cells were induced with 1μM staurosporine (**A**) or 50μM etoposide (**B**), whole cell extracts were prepared at the indicated time points. 30μg/lane whole cell extract was subjected to SDS-PAGE (12.5% minigel), transferred to nitrocellulose and incubated with 68AB/fragment 1 anti-caspase 2 polyclonal antibody at 1:300 dilution The positions of the proenzyme form of caspase 2 (48kDa) and activated enzyme are indicated by arrows. The time of preparation (in hours) of lysates is indicated at the top of each panel. M: prestained molecular weight standards indicated in kDa.

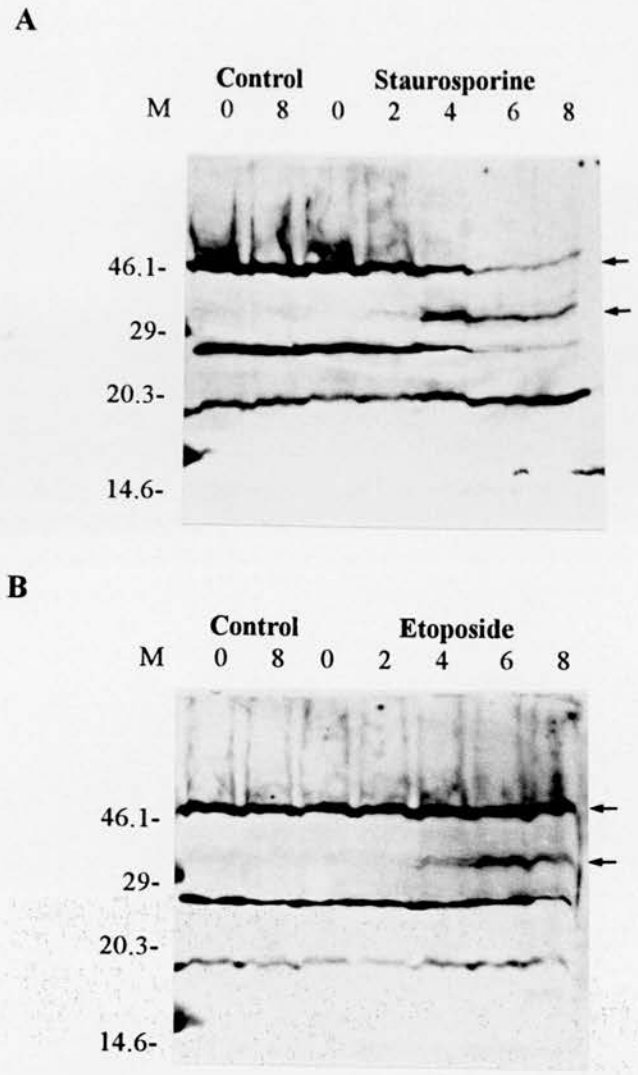

Figure 3.35. Immunoblot analysis of caspase 2 levels in staurosporine and etoposide treated Jurkat cells. Jurkat cells were induced with 1μM staurosporine (**A**) or 50μM etoposide (**B**), whole cell extracts were prepared at the indicated time points. 30μg/lane whole cell extract was subjected to SDS-PAGE (12.5% minigel), transferred to nitrocellulose and incubated with BX08/fragment 2 anti-caspase 2 polyclonal antibody at 1:300 dilution The positions of the proenzyme form of caspase 2 (48kDa) and activated enzyme are indicated by arrows. The time of preparation (in hours) of lysates is indicated at the top of each panel. M: prestained molecular weight standards indicated in kDa.

Western blotting analysis with the prodomain/fragment 1 anti-caspase 2 antibody 68AB shows clear consumption of procaspase 2 in staurosporine induced Jurkat cells at the 4 hour time point (Figure 3.33.A). Procaspase 2 levels are further reduced at

the 6 and 8 hour time points and this reduction in procaspase 2 levels closely correlated with the appearance of an additional band at approximately 32kDa, which was already visible at 2 hours and most likely represents a caspase 2 cleavage intermediate. This 32kDa band probably consists of the prodomain and the large subunit since previous results have shown that this antibody does not cross-react with the large or the small subunit (see Figure 3.11.) but only detected the prodomain. In etoposide treated Jurkat cells the antibody also detects this 32kDa processing intermediate of caspase 2 at 4 hours even though procaspase 2 levels, similarly to procaspase 3 levels, are not markedly reduced (Figure 3.34.B). The antibody also detected two additional protein bands at approximately 29kDa and 18kDa, which are previously identified non-specific bands and are not part of the caspase 2 locus (see Figure 3.15).

Western blotting analysis with the small subunit/fragment 2 anti-caspase 2 antibody BX08 also showed clear consumption of procaspase 2 in staurosporine induced Jurkat cells at the 4 hour time point and further reduction of procaspase 2 levels at 6 and 8 hours (Figure 3.35.A). In etoposide treated Jurkat cells no clear consumption of procaspase 2 was visible (Figure 3.35.B), however, induction of apoptosis with both inducing agents led to the appearance of a faint 32kDa band which is most likely an intermediate caspase 2 processing product. Since this polyclonal antibody was raised against the small subunit of caspase 2 and was shown to not cross-react with other parts of the caspase 2 molecule and four other caspases (Figures 3.11. and 3.12.) this 32kDa processing intermediate of caspase 2 is likely to consist of the large and the small subunit. In both etoposide and staurosporine treated Jurkat cells the antibody failed to detect the small subunit of caspase 2. The antibody also detected an additional protein band of 18kDa in Jurkat extracts which was previously detected by both prebleed and testbleed sera and is thus most likely not related to caspase 2 (see Figure 3.15.).

Two conclusions can be drawn from these results. First, all four non-commercial anti-caspase 2 antibodies gave different results from the Transduction Laboratories monoclonal anti-caspase 2 antibody, clearly indicating consumption of the caspase 2 proenzyme, the appearance of processing intermediates and in some cases, the

appearance of active enzyme subunits. Second, processing of procaspase 2 occurred in distinct steps with kinetics similar to that of procaspase 3 consumption and the cleavage of PARP (see Figure 2.29.). All of these antibodies were raised against recombinant caspase 2 fragments and detected protein bands of the correct size. In contrast, the Transduction Laboratories antibody failed in all of these aspects, binding to a protein of lower molecular weight (46kDa instead of the predicted 48kDa) (Figure 3.18) and failing to detect active enzyme subunits and proenzyme consumption (Figure 3.31.).

In summary, the experiments in this section have shown that caspase 2 is cleaved during apoptosis of Jurkat and Priess cells induced by three different stimuli. The kinetics of cleavage are close to those of caspase 3 activation and cleavage of the caspase 3 substrate PARP. In contrast, the protein recognised by the 'anti-caspase 2' antibody marketed by Transduction Laboratories is not proteolytically cleaved. Previous reports that caspase 2 remains inactivated during apoptosis of these and other cell types derive exclusively from experiments using this monoclonal antibody. These earlier reports might have suggested the need that caspase 2 operates within selective apoptotic pathways only, or is sequestered within cells so that it is generally inaccessible to cleavage during the caspase-driven effector phase of apoptosis. Although this postulate was interesting, the experiments described here show that it is unnecessary: caspase 2 is cleaved with other caspases and their substrates in apoptosis induced by a variety of stimuli.

4. DISCUSSION

The aim of this thesis was to explore the role of caspase 2 in apoptosis by using specific anti-caspase 2 polyclonal antibodies and to determine the distribution of caspase 2 expression using an immunohistochemical approach. Using bacterially expressed recombinant caspase 2 polypeptides two anti-caspase 2 polyclonal antibodies were successfully raised and the specificity of those antibodies to the prodomain and the small subunit of caspase 2 was confirmed. Both antibodies proved unsuitable for use as immunohistochemical and immunocytochemical reagents and cell type- and differentiation-specific patterns of caspase 2 expression were therefore not established. The antibodies, however, proved to be valuable tools in Western blotting and established that caspase 2 is activated relatively early in apoptosis induced by etoposide, Fas activation and staurosporine. Information obtained from immunohistochemistry or Western blotting using the Transduction Laboratories anti-caspase 2 monoclonal antibody was proved irrelevant to the study of caspase 2, as the protein detected by this antibody, although showing interesting cytological associations, was shown not to be caspase 2.

4.1. PRODUCTION OF CASPASE 2 ANTIBODIES

The data presented above show that of the three polypeptides used as immunogens two led to the production of satisfactory antibodies. Fragment 3 from the large caspase 2 subunit was produced in inadequate quantities and was heavily contaminated by bacterial proteins. Fragment 1/68AB (prodomain) and fragment 2/BX08 (small subunit) were satisfactorily induced and gave rise to caspase 2 specific polyclonal antibodies suitable for use in Western blotting. The fragment 2 antibody BX08 is also suitable for use in ELISA analysis, however, the potential cross-reactivity to the small subunit of caspase 4 needs to be considered. The rabbits immunised with caspase 2 fragment 1 and fragment 2, showed a strong immune response to the recombinant caspase 2 immunogen. The resulting antibodies appear to have a relatively low affinity to endogenous caspase 2, but using an adapted Western blotting protocol, both antibodies successfully detected procaspase 2 and two different processing intermediates of 32kDa. Fragment 2/small subunit antibody BX08 failed to detect the small subunit of the active enzyme. This may be attributed to the

low affinity of the antibody and to low levels of active enzyme due to short half live in cells or to technical factors that modulate effective transfer of small fragments in the semi-dry transfer system used.

Both antisera bound non-specifically to one (BX08) or two (68AB) additional cellular proteins. These non-specifically bound proteins are evident using preimmune sera and may contribute to the unsuitability of the antisera for immunocytochemistry and immunohistochemistry. Other reported caspase 2 polyclonal antibodies, raised against histidine tagged recombinant caspase 2 fragments, also show non-specific binding (Li *et al*, 1997) and their application in Western blotting demonstrate similar weak antibody affinity to endogenous caspase 2. The two non-commercial anti-caspase 2 polyclonal antibodies provided by D.W. Nicholson and J.Yuan were unable to detect endogenous caspase 2 in immunohistochemical analysis (Yuan) or had been not tested (Nicholson). Both antibodies showed, similar to the fragment 1 and 2 anti-caspase 2 antibodies, a high affinity to recombinant caspase 2 polypeptides in particular to the immunogen. In contrast, affinity of both of these antibodies to endogenous caspase 2 was relatively weak and was further dramatically reduced after freeze/thawing of the antibodies.

Recombinant bacterial proteins are, however, widely used as antigens in non-commercial and commercial anti-caspase antibodies, including antibodies to caspase 1 and 3. One way to increase the affinity of antibodies for an antigen and to reach higher titers of antibody are multiple rounds of injections. Repeated injections of rabbits (New Zealand white females) with bacterially expressed recombinant caspase 3 protein resulted in a non-commercial polyclonal anti-caspase 3 antibody with high affinity to endogenous caspase 3, suitable for both immunoblot and immuhistochemical analysis (Krajewska *et al*, 1997). The rabbits were injected with 200μg recombinant protein and then boosted seven times. The anti-caspase 2 polyclonal antibodies (fragment 1 and 2) were, however, boosted only three times after the initial immunisation. Repeated injections of the rabbits with recombinant caspase 2 fragments might have increased the affinity of the anti-caspase 2 antibodies which in turn could have affected the sensitivity in both immunoblot and immunohistochemical analysis. Additionally purification of the recombinant proteins

to homogeneity resulting in an immuneresponse only to recombinant caspase 2 polypeptides, could have potentially increased affinity the to endogenous caspase 2. Multiple runs of the eluate fractions over the affinity columns together with increased washing of the columns prior to protein elution could have led to purer recombinant proteins.

Alternatively some properties of the caspases themselves could influence the affinity of antibodies to members of the caspase family. The caspase family is a group of enzymes highly conserved across species with a variety of homologues identified in mouse, hamster, rat, guinea pig, nematode and insects (Kumar *et al*, 1994; Casano *et al*, 1994; Wang *et al*, 1997; Ni *et al*, 1997; VandeCraen *et al*, 1997; Yuan *et al*, 1993; Song *et al*, 1997; Fraser *et al*, 1997; Ahmad *et al*, 1997). Caspases from other species share approximately 30% amino acid identity and up to 60% amino acid similarity with the human caspases, with highest region of homology in the amino acid sequences that form the active enzyme and complete conservation of the active site. Recent two-dimensional affinity blot analysis of active caspases present in apoptotic cell extracts of various human cell lines demonstrated that multiple caspase species are present in apoptotic cells with as many as five different caspase 3 species identified in apoptotic Jurkat extract (Faleiro *et al*, 1997; Martins *et al*, 1997). Some of the caspase 3 species found are cleaved at distinct sites and differ in their molecular weight, suggesting multiple or alternative processing of these caspases and/or post-translational modifications of the processed subunits. However, other species differed only in their isoelectric points, suggesting post-translational modifications. Potential covalent modifications include acetylation, glycosylation, hydroxylations, methylations, phosphorylations, and ADP-ribosylations. Some of these modifications could modulate the activity and alter structural properties of the caspases. No published data about the status of post-translational modifications of individual caspase family members is currently available, two-dimensional analysis does, however, suggests the presence of side chain modifications. Expression of recombinant proteins in bacteria will result in unmodified recombinant proteins lacking post-translational modifications, whereas expression of recombinant proteins in insect cells, able to carry out side chain modifications, could result in recombinant

proteins with higher resemblance to the endogenous protein, thereby potentially increasing immunodetection of the endogenous protein by the antibody.

4.2. SPECIFICITY OF THE TRANSDUCTION LABORATORY MONOCLONAL ANTIBODY

The Transduction Laboratories anti-caspase 2 monoclonal antibody was raised against a 19.5kDa recombinant caspase 2 fragment, although, no information on the identity of the fragment, the immunisation scheme, the antibody epitope or specificity were available on request. The evidence from several independent experiments carried out as part of this thesis with this antibody clearly shows that this antibody does not bind to caspase 2. In immunoblot analysis of seven human cell lines the antibody detected a protein band of approximately 45-46kDa, a protein with an approximately 2kDa lower molecular weight than the predicted molecular weight of procaspase 2 of 48kDa (Yuan *et al*, 1994; Xue *et al*, 1996). Comparative immunoblot analysis with the Santa Cruz Biotechnology commercial polyclonal anti-caspase 2 antibody, which detected a protein band of approximately 48-50kDa further questions the claimed specificity of the Transduction Laboratories antibody.

Western blotting analysis of recombinant caspase 2 large and small subunit and the recombinant caspase 2 fragments with the Transduction Laboratory antibody further seriously warrants re-evaluation of the antibody specificity, as the antibody failed to detect any of the recombinant proteins analysed, whereas the two non-commercial polyclonal antibodies (D.W. Nicholson and J. Yuan) and the fragment 1 and fragment 2 antibodies detected recombinant caspase 2 fragments and subunits.

Immunoblot analysis with the Transduction Laboratory monoclonal anti-caspase 2 antibody further revealed no significant change of quantity or molecular weight of the protein detected during apoptosis in Jurkat and Priess cells in response to three different stimuli under conditions of activation of apoptosis. Under the same conditions the two non-commercial polyclonal anti-caspase 2 antibodies (D.W. Nicholson and J. Yuan) show consumption of procaspase 2 in both cell lines to a 32kDa band and an approximately 18kDa band and polyclonal antibodies 68AB and BX08 show clear consumption of procaspase 2 and the appearance of the cleavage intermediate. These observations with all four polyclonal anti-caspase 2 antibodies thus firmly establish that caspase 2 is activated during apoptosis in Jurkat and Priess

cells and contradict the conclusion from previous immunoblotting results of the same extracts with the monoclonal Transduction Laboratory antibody. Other laboratories using the Transduction Laboratories monoclonal anti-caspase 2 antibody also failed to show consumption of the detected protein and were also unable to detect any lower molecular weight proteins corresponding to the active enzyme subunits in HL60 cells (a human promyelocytic cell line) induced with etoposide (Martins *et al*, 1997), with staurosporine (Han *et al*, 1996), or treated with anisomycin or geranylgeraniol (Polverino *et al*, 1997). When blots of the same HL60 apoptotic lysates treated with either anisomycin or geranylgeraniol were, however, subsequently probed with a goat polyclonal anti-caspase 2 antibody from Santa Cruz Biotechnology, a band with similar mobility to caspase 2 decreased in intensity over time with a concomitant increase in intensity of a band of approximately 12kDa, corresponding to the predicted molecular weight of the small subunit of caspase 2. Similar results were obtained using a Santa Cruz Biotechnology rabbit polyclonal antibody against caspase 2, although additional non-specific bands were observed. Following stimulation with anti-Fas antibody caspase 2 activation was also detected in HL60 cells with the goat anti-caspase 2 polyclonal antibody (Polverino *et al*, 1997).

Published data using the Transduction Laboratory monoclonal antibody suggest that caspase 2 is uncleaved during apoptosis with a variety of stimuli and a time when other caspases are activated and their substrates are cleaved. This result would have been of great interest suggesting a complex biology for caspase 2 with potentially stimulus specific activation. The data presented here, however, show that these results have a different interpretation: The Transduction Laboratories antibody does not detect caspase 2. Paradoxically it binds to protein, apparently abundant in the cytoplasm, which remains stable late in apoptosis.

4.3. BIOLOGICAL FUNCTION OF CASPASE 2

Caspase 2 is a member of the caspase protease family, the effector proteases in the apoptotic pathway. It shares a high sequence similarity with other caspase family members and contains three caspase specific aspartic acid cleavage sites at which the proform of caspase 2 is cleaved to form the active enzyme, consisting of 12 and 18kDa subunits (Xue *et al*, 1996). It is present in several cell types, including T-cells, B-cells and monocytes (Tamura *et al*, 1995; Han *et al*, 1996). Structurally caspase 2 has, similar to caspase 9, a long prodomain. This prodomain of caspase 2 contains protein motifs that mediate association with similar motifs in the NH_2-terminus of the linker molecule CRADD/RAIDD, which shares significant sequence identity with caspase 2, caspase 9 and CED-3, and also binds to the death domain of RIP. This suggests that CRADD/RAIDD and caspase 2 may play a role in the transduction of Fas- and TNF-receptor apoptotic signaling pathway and that caspase 2 might be activated in response to Fas- and TNF mediated apoptosis (Ahmad *et al*, 1997; Duan *et al*, 1997). The inability of mutant caspase 2 or various putative dominant-negative versions of RAIDD to block TNFR-1 induced apoptosis, however, points to an alternative TRADD-FADD-FLICE (caspase 8) pathway (Duan *et al*, 1997).

It has been argued that long prodomain caspases, based on their interaction with signal transduction molecules via their prodomain, may be the first caspases to be activated which in turn activate other short prodomain caspases (Harvey et al, 1997). Unlike other caspase family members, including 3, 8 and 10, procaspase 2 has, however, been shown to be unable to activate any procaspases other than its own precursor proenzyme (Ahmad *et al*, 1997). *In vitro* caspase 2 has been shown to be cleaved by caspase 3 (Li *et al*, 1997) and recombinant caspase 8, which directly cleaves various recombinant caspases, was found to cleave caspase 2 efficiently only at a relatively late stage (1-2 hours) in the presence of cytosolic extract, suggesting the requirement for an additional cytosolic component to mediate the activation of caspase 2 by caspase 8 (Muzio *et al*, 1997). The observation that caspase 2 is activated in CTL-mediated apoptosis, but is not activated by granzyme B directly, also suggests that other factors are required for caspase 2 activation in perforin-granzyme B killing (Li *et al*, 1997). Currently unpublished reports of caspase 2/Nedd2

knock-out mice show that these mice have no apparent phenotype suggesting either redundancy in the apoptotic pathway or no central role in the initiation of apoptosis for caspase 2.

Immunological studies of this thesis show that procaspase 2 is activated in Jurkat and Priess cell apoptosis induced by etoposide, anti-Fas and staurosporine, detectable by a reduction in procaspase levels, and the appearance of 32kDa cleavage intermediates and the large subunit. This cleavage and activation of procaspase 2 occur in distinct steps through a processing intermediate which appears at around the same time as caspase 3 activation and cleavage of the well characterised caspase substrate PARP.

Activation of caspase 2 has been reported in other cell lines including Jurkat and HeLa cells induced with anti-Fas antibody, staurosporine and TNFα (Li *et al*, 1997) and THP.1 cells induced with etoposide (MacFarlane *et al*, 1997), in which procaspase 2 is processed in distinct steps. A 32-33kDa doublet, which consists of the large and the small subunit and the linker of caspase 2, appears simultaneously to caspase 3 activation and PARP cleavage, but caspase 2 cleavage to the active subunits occurs only 2-3 hours later than caspase 3 activation (Li *et al*, 1997). Data from this thesis, however, suggest that two different processing intermediates are formed, one consisting of the prodomain plus the large subunit while the other one is formed by the large and the small subunit and the linker region. Taken together, these results suggest that the cleavage events that reduce procaspase 2 to its two active subunits can occur in any order.

The mouse homolog of caspase 2, Nedd2, has also been shown to be activated in three mouse cell lines (NIH-3T3, Mo7e and BL30A cells) following induction of apoptosis by interleukin 3 and serum withdrawal, etoposide treatment and γ-irradiation (Harvey *et al*, 1996; Harvey *et al*, 1997). Studies of caspase 2 activation in THP.1 cells induced with etoposide give no indication to the time of activation of procaspase 2 in respect to other caspases or cleavage of caspase substrates (MacFarlane *et al*, 1997). Studies of Nedd2 activation in two mouse cell lines, however, suggest that Nedd2 is activated in response to a variety of apoptotic stimuli and that this activation occurs earlier than caspase 3 activation. Recent reports from immunoblot and affinity labeling experiments, which indicate that as many as nine

caspase genes are simultaneously expressed in certain leukaemia cell lines and that at least four caspases, including caspase 2, are activated during apoptosis, (MacFarlane *et al*, 1997; Martins *et al*, 1997) leave unclear whether all caspases participate in apoptotic events.

Observations in different cell lines induced to undergo apoptosis by a variety of different stimuli thus all demonstrate activation of caspase 2 in apoptosis. Both the mechanism of caspase 2 activation, the exact identity of the upstream molecule leading to active caspase 2 and the kinetics of this activation in relation to other caspases remains unclear. Identification/development of natural or synthetic substrates is required to further elucidate the role of caspase 2 activation in apoptosis.

5. MATERIALS AND METHODS

5.1. DESIGNING CASPASE 2 SPECIFIC POLYPEPTIDES AS ANTIGENS

5.1.1.Sequence alignment of caspase family members

The protein sequence of four caspases, human caspase 1/ICE, 2/ICH-1, 3/CPP32/Yama/apopain and *C. elegans* CED3 were retrieved from the Swiss-Prot protein databank. The four protein sequences are entered into the protein database under the following accession numbers caspase1/ICE: P42573, caspase 2/ICH-1L/S:P42575; caspase 3: P42574. Multiple sequence alignment of the four protein sequences was carried out by ClustlalW multiple sequence alignment programme applying the incorporated default setting options. ClustalW is accessible for registered users through HGMP, Hinxton.

5.1.2. Caspase 2 antibody strategy

Amino acid sequences of four caspases were compared and conserved amino acid sequences between the four proteases were identified. Stretches of amino acid sequence of least homology to other known caspases and specific for caspase 2 proenzyme, the large p18kDa and the small p12kDa subunit were chosen as antigens to raise caspase 2 specific polyclonal antibodies.

5.2. RNA EXTRACTION

5.2.1.Treatment of water and equipment for use with RNA

Diethyl pyrocarbonate (DEPC) treated water for RNA work was prepared by adding DEPC at a final concentration of 0.1% v/v to deionised double distilled water (ddH_2O) in a fume hood and small batches were autoclaved. DEPC treated ddH_2O was used for the making of all solutions.

A separate set of pipettes designated 'RNA work only' was used for all RNA work, other items of equipment were either guaranteed 'RNAse-free' by the manufacturer or immersed over night in DEPC treated water and autoclaved to prevent RNA degradation by contaminating RNAse.

5.2.2. Isolation of total RNA from HeLa cells

TRIzol Reagent (Life Technologies) was used for the preparation of RNA from HeLa cells according to the manufacture's instructions. TRIzol is a mono-phasic solution of phenol and guanidine isothiocynate, and is based on the method developed

by Chomczynski and Sacchi (1987). Briefly, 6 x 10^6 HeLa cells were washed twice in 5ml of phoshate buffered saline (PBS) (Appendix 1) and were mixed with 1ml of TRIzol reagent, lysed by repetitive pipetting and incubated at room temperature for 5 min. 0.2ml of chloroform was added, the mixture inverted several times and then incubated at room temperature for 2-3 min. The sample was centrifuged at 13000rpm in a bench top microfuge (Micro Centaur, MSE) for 15 min at 4°C and the aqueous phase was recovered. 0.5ml propan-2-ol was added to precipitate the RNA and RNA was recovered by centrifugation at 1300rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 1ml of 75% ethanol in DEPC ddH_2O, and centrifuged at 6500rpm for 5 min at 4°C. The ethanol was discarded and the RNA pellet was dried under the vacuum (Edwards, High voltage vacuum pump) for 3-4 min, prior to resuspension in 50µl of DEPC ddH_2O. The concentration of total RNA was estimated by ultraviolet spectrophotometry at 260nm using a Genequant II RNA/DNA Calculator (Pharmacia).

5.3. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

5.3.1. Reverse transcription

First strand complementary DNA (cDNA) was synthesised from total RNA using an RNAse H- Moloney Murine Leukaemia Virus Reverse Transcriptase (Superscript II, Life Technologies) and an oligo (dT) primer (Boehringer). A reaction mixture of 1-2µg of total RNA, 100ng primer $(dT)_{15}$ and 11µl DEPC ddH_2O, was overlayed with 19µl paraffin oil (Boots), heated to 70°C for 5 min and quickly chilled on ice for 5 min. The reaction mixture was then made up to 20µl by adding 4µl of 5x first strand buffer, 2µl 0.1M dithiothreitol (DTT), 1µl of 10x mixed dNTP stock solution (Appendix 1) and 200 units RNAse H- Superscipt, was briefly microfuged, incubated on ice for 10 min before being placed on a thermal heating block (Ommni-gene, Hybaid) on the following programme:

37°C for 10 min
42°C for 60 min
50°C for 10 min
94°C for 10 min

Reactions were placed on ice, diluted with DEPC ddH_2O to a final volume of 100µl and stored at -20°C. Control reactions were carried out to which no RNA was added.

5.3.2. RT-PCR for human caspase 2

Total RNA from HeLa, a human cervix epitheloid carcinoma cell line, was isolated and reverse transcribed as described above and subjected to PCR with custom-manufactured and cartridge-purified oligonucleotide primers (Cruachem). Primers were designed to synthesise the whole sequence of caspase 2, oligonucleotide sequence corresponding to a NH_2-terminal protein prodomain fragment (fragment 1), a COOH-terminal small subunit fragment (fragment 2) and large subunit fragment (fragment 3). The primers are shown below:

Whole sequence
Forward: 5'> TATACCATGGCCGCTGACAGGGGA <3'
Reverse: 5'> ATACTCGAGAGGGTGTCCTGGGAACAG <3'

Fragment 1
Forward: 5'> GTAACCATGGATCCTCATCATCAGGAA <3'
Reverse: 5'> TATCTCGAGGCCAGAAAGGGTGGTGAGCAA <3'

Fragment 2
Forward: 5'> TATACCATGGAGACTGATCGTGGGGTT <3'
Reverse: 5'> ATACTCGAGAGTGCTGCAGTATTCAGA <3'

Fragment 3
Forward: 5'> ATTACCATGGAAGATGGTCCTGTCTGC <3'
Reverse: 5'> TAACTCGAGCTGAAAAACCTCTTGGAG <3'

All primers incorporate an Nco I (forward) and a Xho I (reverse) restriction endonuclease site to permit easy cloning. The PCR reaction mixture contained the following components:

10µl of 10x Thermophilic buffer
3µl of 50mM magnesium chloride
2µl mixed dNTP stock solution (Appendix 1)
1µl (100ng) of each primer
2.5 Units Taq (Thermus aquaticus) DNA polymerase (Life Technologies)
made up to a final volume of 100µl with autoclaved ddH_2O

The mixture was overlaid with 75µl paraffin oil and amplification was carried out on a thermal cycler (OmmniGene, Hybaid) according to the following programmes:

PCR programme for whole sequence of caspase 2:

Step 1, repeated once	94°C, 3 min
	65°C, 1 min
	72°C, 1 min
	94°C, 30 sec
Step 2, repeated 30 times	65°C, 20 sec
	55°C, 30 sec
	94°C, 30sec
Step 3, repeated once	70°C, 1 min
	72°C, 5 min
	25°C, 10 sec

PCR programme for caspase fragments 1-3:

Step 1, repeated once	94°C, 3 min
	65°C, 1 min
	72°C, 3 min
	94°C, 30 sec
Step 2, repeated 30 times	65°C, 20 sec
	72°C, 30 sec
	94°C, 30 sec
Step 3, repeated once	70°C, 1 min
	72°C, 5 min
	25°C, 10 sec

10µl of RT-PCR products were analysed on a 1% agarose gel in 1x Tris-borate EDTA (TBE) (Appendix 1) as described below. Products were stored promptly at -20°C.

5.4. ANALYSIS AND PREPARATION OF PLASMID DNA

5.4.1. DNA quantification

DNA was quantified by spectrophotometry measuring the optical density (OD) of 1:100 diluted DNA solutions at a wavelength of 260 nm in a Genequant II RNA/DNA Calculator (Pharmacia) in which an OD_{260} of 1 is equivalent to a concentration of 50µg/ml of double stranded DNA. Alternatively DNA quantities were determined by comparing the fluorescent intensity of DNA samples pipetted onto ethidium bromide petri dish plates under ultraviolet (UV) light to known

quantities of DNA standards. DNA standards in the range of 10-250ng/ml were made up with either Kilobase DNA ladder (Life Technologies) or Marker V (Boehringer) diluted in ddH_2O. Petri dishes were prepared of 1% agarose gel containing 5μg/ml ethidium bromide; samples pipetted onto the petri dish were left for 10-15 min for absorption and then placed on an UV transilluminator (Herolab) for estimation of DNA concentration. DNA purity was assessed on the Genequant by measurement of the $OD_{260/280}$ ratio.

5.4.2 Restriction endonuclease digestion of DNA

The DNA digestions were set up in sterile microfuge tubes (Eppendorf) containing a mixture of ddH_2O, the appropriate restriction endonuclease and the manufacturer's recommended buffer of the correct ionic strength. All restriction enzymes were purchased from Boehringer, stored at -20°C and kept in a bench top Stratacooler (Stratagene) while in use. The required amount of DNA was digested in a reaction mix containing the restriction endonuclease to an excess of the recommended activity for the amount of DNA to be digested (1 unit of enzyme being the amount required to digest 1μg of DNA to completion in 1 hour) in ddH_2O containing 10x stock buffer, diluted 1:10 in the final reaction volume. Digestion was carried out for at least one hour at 37°C or as directed.

5.4.3. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was carried out to analyse PCR products and restriction digests, to characterise plasmids and to purify restriction fragments for further cloning steps. Preparation of gels and electrophoresis was carried out in Bio-Rad MiniSub Cells or Anachem Origo H3 gel tanks as described by Sambrook et al (1989). 0.1 volume of gel loading buffer (Appendix 1) was added to samples and loaded into wells submerged in TBE electrophoresis buffer. Kilobase DNA ladder (Life Technologies) or Marker V (Boehringer) was run in parallel and used for sizing DNA fragments.

5.4.4. Isolation of DNA fragments from agarose gels: Gel purification

Digested DNA fragments were run out on agarose gels, visualised by UV transillumination and excised from the gels in the smallest possible volume. Fragments

were purified using the GlassMAX DNA Isolation Spin Cartridge System (Life Technologies) according to the manufacture's instructions.

5.4.5. Purification of DNA fragments: Removal of oligonucleotides

To remove primer dimers formed in PCR reactions from PCR products, reaction mixtures were purified on Quick Spin G-50 Sephadex Columns (Boehringer Mannheim) according to the manufacture's instructions. Double stranded DNA fragments of size up to 72 base pairs were retained in the column resulting in purification of larger DNA fragments. All PCR products were designed with incorporated restriction XhoI and NcoI endonuclease sites, which after a restriction digest with the two enzymes, gave compatible ends with the cloning vector. To remove oligonucleotides from restriction digests, in order to prevent religation of digested oligonucleotides in ligation reactions, restriction digests of PCR products were column purified.

5.4.6. Ethanol Precipitation of DNA

The procedure was carried out as described in Sambrook et al, 1989. Plasmid DNA was precipitated by the addition of 1/10 volume of 3M NaAc and 2 volumes of ice-cold absolute ethanol (98% EtOH). If the DNA quantity was very small, i.e. less then 50ng, 1μg of glycogen was added as an inert carrier. The mixture was chilled on dry ice until viscous or alternatively incubated at -70°C for 30 min. The DNA was pelleted in a microfuge at 12000g at 4°C for 10 min, the EtOH was discarded and the pellet was washed with ~0.5ml 70% EtOH. The supernatant was removed and the DNA pellet was air dried and resuspended in TE, pH 8.0 (Appendix 1).

5.4.7. Ligation of caspase 2 DNA fragments

Following fragment isolation appropriate amounts of fragment for a ligation reaction were calculated by the following formula according to Sambrook et al, 1989:

$$x \text{ ng PCR product} = \frac{(y \text{ bp PCR product})(50\text{ng vector})}{\text{size in bp of vector}}$$

Generally 50ng of vector was ligated to approximately an equimolar or 1:2 amount of insert DNA. RT-PCR products were cloned by direct insertion into pET23d vector (3663 bp) (Novagen). Maxiprepped circularised vector DNA was linearised in a

restriction enzyme digestion with XhoI and NcoI to provide compatible ends for the cloning of the PCR *caspase 2* fragments. Both restriction enzymes cut the vector at a single site releasing a fragment of 80bp which was further cleaved at a single site by treatment with the restriction enzyme EcoRI. In order to prevent religation in subsequent ligation reactions, the linearised vector was purified from the cut out fragments on G50 Sephadex column. The PCR amplified *caspase 2* fragments were also digested with the restriction enzymes NcoI and XhoI to provide compatible cloning ends followed by G50 Sephadex column purification. 1μL of pretreated PCR product was added to 50ng of linearised pET23d in a 10μl ligation reaction containing 3 units T4 DNA ligase and 1μL l0x ligase buffer (Promega). A control ligation containing no insert DNA was always included to assess the extent of self-ligation. The reaction components were assembled fresh each time and the reaction was incubated at 15°C for a minimum of 4 hours or left over night.

5.4.8. Transformation of competent E.coli cells

Transformation reaction were carried out with commercially produced competent *E. coli* cells. Competent cells were defrosted on ice and aliquoted into pre-chilled 1.5ml microfuge tubes. Unused aliquots were snap frozen in a dry ice methanol bath and stored at -70°C. For each transformation reaction 1μL of the ligation reaction was gently mixed with 20μL XL1-BlueMRF' competent cells (Stratagene) and incubated on ice for 30 min. Cells were then heat shocked for 45 sec in a 42°C water bath and placed back on ice for 2 min. 80μL of S.O.C. medium (Appendix 1), pre-warmed to room temperature was added to each vial and cells were incubated in a shaking incubator at 225 rpm at 37°C for 1 hour to allow expression of β-lactamase and to confer antibiotic resistance. Controls were provided by parallel transformation with no DNA (negative control) or with pUC19 DNA (positive control). Transformed cells which conferred ampicillin and chloramphenicol resistance were then spread onto selective medium plates (disposable 100mm bacterial culture dish, containing L-broth (LB) supplemented with 1.2% Bactoagar, containing 34μg/ml chloramphenicol and 50μg/ml ampicillin (Appendix 1). If, in the case of XL1-BlueMRF' cells, blue white selection was to be used, L-Amp/Choramp plates were spread with 100μl 20mM IPTG (isopropyl-β-D-thio-galactopyranoside, Stratgene) and 100μl 0.5% X-Gal (5-

bromo-4-chloro-3-indoyl-β-D-galactopyranoside, Boehringer) and incubated at 37°C for 30 min prior to spreading of the transformed cells.

Plates were incubated over night at 37°C. In case of blue white selection plates were placed at 4°C for a minimum of 2 hours to allow the blue color development. White (or light blue) colonies were picked as they were an indication of the disruption of the lacZ α-peptide reading frame in most cases by insertion of the PCR product and therefore contained recombinant plasmid.

For protein expression the recombinant pET23d plasmid with the correctly inserted *caspase 2* fragment was transformed into *E. coli* protein expression strain BL21 (DE3) pLysS as described above. Plates with colonies for protein expression were stored up to 10 days at 4°C after which new transformations had to be set up. Stocks of transformed cells could not be frozen due to lysozyme expression in BL21pLysS cells.

5.4.9. Small scale preparation of plasmid DNA (Mini-preps)

Plasmid DNA was purified form bacterial cultures for restriction digest analysis using the Wizard Minipreps DNA purification systems (Promega). Single colonies were picked from L-Amp and inoculated into 5ml of LB medium containing the appropriate antibiotic in Falcon 2059 tubes (Becton Dickinson) and were incubated over night at 37°C in an orbital shaker at 225rpm. In brief, 1.5ml of cells were transferred into a microfuge tube and the bacteria were pelleted at 13000g for 2 min. The culture medium was discarded and the cell pellet was resuspended in 200µl cell suspension solution. Cells were lysed by addition of 200µl cell lysis solution, resulting in denaturation of bacterial protein and chromosomal and plasmid DNA, and the solution was neutralised from strong alkaline conditions by addition of 200µl neutralising solution. Addition of neutralising solution results in rapid renaturation of circular plasmid DNA which is separated from the bulk of denatured proteins, chromosomal DNA and the SDS by centrifugation at 13000g for 5 min. The cleared lysate was transferred into a clean microfuge tube and the plasmid DNA was purified on Wizard minicolumns using a vacuum manifold according to the manufacture's instructions. Purified plasmid DNA was resuspended in 50µl of TE, pH 8.0 (Appendix

1). 2-3μL of this was used for one restriction digest and was analysed by gel electrophoresis.

5.4.10. Large scale preparation of plasmid DNA (Maxi-preps)

For large scale preparation of plasmid DNA, 500ml of LB medium (Appendix 1), containing the appropriate antibiotic was inoculated with 20μl of miniprep culture and incubated at 37°C over night (no longer than 16 hours) in a shaking incubator at 225rpm. Plasmid DNA was purified by anion exchange column chromatography supplied in kit form by Qiagen according to the manufacture's instructions (Tip-2500). The concentration and purity of the DNA was estimated as described above.

5.4.11. DNA sequencing

Maxi-prepped plasmid DNA was sequenced according to Sanger *et al*, 1977, using a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical) in order to confirm correctness of the oligonucleotide sequence and an in frame insertion of the cloned fragment. 5μg of double stranded DNA was diluted in 45μl ddH_2O and denatured with 5μl of 2M NaOH, 2mM EDTA and incubated for 5 min at room temperature. The solution was neutralised with 5μl NH_4Ac, pH 4.6 and the DNA was precipitated at -70°C for 30 min with 185μl of absolute ethanol. DNA was pelleted by centrifugation in a bench top microcentrifuge at 13000g for 10 min at 4°C and the pellet was washed once with 200μl of ice-cold 70% ethanol, centrifuged as above for 2 min and dried under vacuum for 5 min. The pellet was resuspended in 6μl ddH_2O for immediate use or stored dry at -20°C for up to one week. The resuspended DNA was annealed to 2pmol of primer (T7 upstream primer and M13 downstream primer) in 1x Sequenase buffer in a final volume of 10μl at 65°C for 2 min, slowly cooled to <37°C in a water bath placed on the bench over 15-30 min, placed on ice for 30 sec, centrifuged briefly and put back onto ice. The annealed template/primer mix was then labelled at room temperature for 3-5 min with 5μCi (1000Ci/mmol) of [α-^{35}S]-dATP in 3.5μl labelling mix plus 2μl of 1:8 diluted Sequenase. 2.5μl of the termination mix for each of the four dideoxyribonucleotides was aliquoted into separate microfuge tubes and heated up to 37°C. 3.5μl of the labelling reaction was added to each tube and the reaction was terminated after 5 min by the addition of 4μl stop solution to each tube. Samples were stored at -20°C. Prior to running on a sequencing gel

samples were denatured by heating to 75°C for 5min. 3µl of each reaction was loaded into wells and run at 70Watts in 1x TBE on a pre-run (~1 hour to reach temperature of 45°C) 6% denaturing polyacrylamide sequencing gel (Appendix 1) using a S2 sequencing apparatus (Life Technologies) and a model 300 microcomputer electrophoresis power supply. At the end of the run the silicon coated (Gel-Slick) small plate was lifted off and the sequencing gel was fixed twice with 10% acetic acid, 10% methanol for 5 min, transferred to a piece of 3MM Whatman paper, covered in plastic film and dried on a model 583 vacuum gel drier (Bio-Rad) for 2 hours at 80°C. The cling film was removed and the dried gel was incubated with Biomax MR film (Kodak) in an autoradiography cassette for a minimum of 18 hours. The film was developed in a Hyperprocessor (Amersham) and analysed on a white light box.

5.5. TARGET GENE EXPRESSION

5.5.1. Induction of target gene expression

For protein expression a single colony was picked from plates up to 10 days old and inoculated 400ml of LB medium containing 50µg/ml ampicillin and 34µg/ml chloramphenicol in a 1l Erlenmeyer flask to ensure sufficient aeration. The culture was incubated with shaking at 37°C and 1ml samples were taken at 1h intervals, analysed on a spectrophotometer (Philips PU8620) at a wavelength of 600nm to monitor the culture growth. Once an OD_{600} between 0.4-1 (ideally 0.6) was reached, on average after about 8 hours, target gene expression was induced by addition of 0.4mM IPTG (Appendix 1). The incubation was continued for 3 hours to allow substantial accumulation of target protein followed by harvesting of the culture.

5.5.2. Detection of target gene expression: Analysis of total cell protein

Immediately before induction a 500µl aliquot of control cell sample was taken. To study the time course of target protein expression after induction, 500µl aliquots of the culture were taken at 1 hour intervals. Cells were collected by centrifugation at 13000g for 2 min, the supernatant was removed and the cell pellet was resuspended in 10mM Tris-HCL buffer, pH 8.0 (Appendix 1) and lysed by addition of 50µl of 2x SDS sample buffer (Appendix 1). DNA was sheared by running the samples through a syringe with a 25 gauge needle and samples were stored at -20°C. Roughly 50µg of

protein (equivalent of ~15µl uninduced cells and ~10µl induced cells) were analysed on 15% SDS-PAGE electrophoresis (see below).

5.5.3. Purification of target proteins: Soluble and insoluble fraction

All solutions including the chromatography resin were obtained in kit from Novagen (pET system). 400ml of induced cell culture was transferred into 50ml blue cap Falcon tubes and was harvested by centrifugation at 3400rpm for 5 min in a Sorvall RT 6000D centrifuge. The supernatant was decanted and the cell pellet was drained and resuspended in 4ml ice-cold binding buffer in a Falcon tube. Cells were lysed by 5 freeze/thaw cycles in a dry-ice methanol bath or liquid nitrogen and a water bath of 16°C with in-between vortexing on a bench top vortexer (Scientific Industries). At the end of the freeze thaw cycles, samples were homogenised with the tight pestle in a Wheaton 15ml homogeniser. To purify soluble target proteins, which remain soluble in the cytoplasm the cell lysate was centrifuged in SWT-60 swing bucket rotor in a Sorvall ultracentrifuge at 39000rpm for 20 min. The supernatant was put through a syringe with a 25 gauge needle and filtered through a 45 micron syringe end filter (Sartorius) to break up any DNA and to prevent clogging of the resin. The resin was prepared according to the manufacture's instructions (Novagen) and column chromatography was carried out as described in the manufacture's manual (Novagen) except twice the amount of all solutions was used due to the increased culture volume. Insoluble proteins were purified under denaturing conditions by addition of 6M urea to all solutions including the resin unless stated otherwise, as described in the manual. Cells were centrifuged as above, the cell pellet was subjected to 5 freeze/thaw cycles and resuspended in 40ml of ice-cold binding buffer without urea. The solution was homogenised as above centrifuged at 13000rpm in a Sorvall SS 34 centifuge for 15 min to collect inclusion bodies and cellular debris while leaving other proteins in solution. The supernatant was discarded and the cell pellet was resuspended in 20ml of binding buffer without urea and the homogenisation and centrifugation was repeated. The pellet was resuspended in 5ml of binding buffer plus 6M urea and incubated on ice for 1 hour to completely dissolve the protein. Remaining insoluble material was removed by centrifugation as described for soluble protein purification. Recombinant proteins were then purified by nickel metal

chelating chromatography as described in the manual. Columns were discarded after five runs of protein purification. Protein concentrations were determined by Bio-Rad protein assay (see below) and purified samples of known protein concentration were concentrated using a plastic disposable ultrafiltration device, Vivaspin 15 (Vivascience) with an exclusion size of 5kDa, as described in the operating instructions.

5.7. PROTEIN ANALYSIS

5.7.1. Protein quantification: Bio-Rad assay

Protein concentration were determined using a Bio-Rad protein assay kit as described in the manufacture's instructions (Bio-Rad) and both micro- and macroassays were carried out. Bovine serum albumin (BSA), diluted to various known concentrations was used as standards. Absorbance was read at 595nm on a Philips PU8620 spectrophotometer using disposable 1ml plastic cuvettes (Bio-Rad) and a standard curve was prepared each time the assay was performed by plotting the OD_{595} reading versus the concentration of the standards. The absorbance of protein samples of unknown concentration was measured and unknown protein concentrations were determined by reading from the standard curve.

5.7.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was carried out according to the Laemlli systems (Laemlli, 1970) and proteins were separated on 7.5%, 12.5% or 15% separating (running) gels.

Table 5.1. Running gel and stacking gels recipes

Running gel final concentration (60ml; for ~10 1mm thick minigels, for 2 1.5mm thick SE600/400 gels)

	5%	7.5%	10%	12.5%	15%
Monomer solution	10ml	15ml	20ml	25ml	30ml
4x running gel buffer	15ml	15ml	15ml	15ml	15ml
10% SDS	0.6ml	0.6ml	0.6ml	0.6ml	0.6ml
ddH_2O	34.1ml	29.1ml	24.1ml	19.1ml	14.1ml
10% Ammonium persulphate	300µl	300µl	300µl	300µl	300µl
TEMED	20µl	20µl	20µl	20µl	20µl

Stacking gel solution (4% acrylamide; for 2 SE600/400 gels, 1.5mm SE600/400 gel solution for ~ 10 minigels)

	0.75mm	1.5mm
Monomer solution	1.33ml	2.66ml
4x running gel buffer	2.5ml	5.0ml
10% SDS	0.1ml	0.2ml
ddH_2O	6.0ml	12.0ml
10% Ammonium persulphate	50µl	100µl
TEMED	5µ	10µl

All stock solutions were made up according to the Hoefer protein electrophoresis guide (Appendix 1). Unstained protein standards were used for Coomassie stained gels (Life Technologies). Pre-stained molecular weight markers in low and high range were used for Western blotting transfer (Life Technologies). TEMED (Tetramethylethylethyenediamine) and bis-acrylamide at a ratio of 29:1 were obtained from Severn Biotechnology. Minigels were poured in a multicasting tank (Bio-Rad) in sets of 10-15 and were stored, wrapped up in cling film, at 4°C up for up two weeks. Large gels were run in a SE 600 Vertical Gel Electrophoresis Unit (Hoefer) and minigels were run on a SE 250 Minigel Electrophoresis Unit (Hoefer). On average 10-20µg of total protein were run on minigels and 50-80µg were run on large gels. Samples were typically boiled for 2 min on a heating block (Techne Dri-Block) and placed on ice until ready for use. Gels were run at 30mAmps per 1.5mm thick gel until the dye reached the bottom of the gel. Gels were removed from the glass plates, the stacking gel was cut off and gels were placed in rapid stain fixing solutions

(Appendix 1) for 10-15 min for a 0.75-1.0mm gel and 30-60 min for a 1.5mm thick gel. Fixing solution was replaced with rapid Coomassie stain (Appendix 1) and was shaken slowly for 2 hours to overnight until bands were visible. Staining solution was replaced with destaining solution (Appendix 1), frequently replaced by fresh solution until the background was clear. To avoid cracking of gels thicker than 0.75mm and over 12.5% acrylamide 2% glycerol was added to the final destain solution. Gels (final gel concentration lower than 12.5%) were dried on a vacuum gel drier (Bio-Rad 583 Gel Drier). Gels were placed onto a sheet of filter paper, the top of the gel was covered with plastic wrap and the gel was dried for 2 hours at 80°C. Alternatively gels were air dried in a drying frame using the Dry Ease Gel Drying System (Novex) according to the manufacture's instructions.

5.7.3. Preparation of human tissue protein extracts

Samples of approximately 50mg of frozen tissue were cut off with a disposable scalpel, transferred to a sterile microfuge tube and thawed on ice in 4 volumes of ice-cold 0.1 M sodium phosphate lysis buffer (Appendix 1). Human soluble tissue extracts were prepared by homogenisation using an Omni EZ Connect homogeniser followed by centrifugation at 15000g in a bench top microfuge for 10 min at 4°C, to remove insoluble material. Protein concentrations were determined as described above and samples were stored at -20°C.

5.7.4. Whole cell protein extract preparation

Protein extracts were prepared from 2 x 10^7 cells, pelleted by centrifugation at 1000rpm for 5min, washed once with PBS and lysed in 500µl cell lysis buffer (Appendix 1). DNA was sheared by putting the extracts through a syringe with a 25 gauge needle. Protein concentrations were determined as described above and samples were stored at 4°C for immediate use or at -20°C for long term storage.

5.8. WESTERN BLOTTING

5.8.1. Conventional tank blotting

Protein transfer from SDS-PAGE gels to membranes was carried out as described in the Protein Electrophoresis Guide (Hoefer) (Protein Electrophoresis Applications Guide, Hoefer Scientific Instruments). In brief, SDS-PAGE gels were pre-equilibrated for 5-15 min in Towbin transfer buffer (Appendix 1). Nitrocellulose

membrane (Hybond ECL, Amersham) and blotting paper were cut to size, were wetted in dH_2O and soaked in transfer buffer for 2-5 min. The transfer sandwich using a Bio-Rad Trans-Blot transfer unit for large gels and a Bio-Rad Mini Protean II unit for small gels was assembled under buffer to minimise air trapping in the following order: Grey or clear site of cassette (facing positive electrode)/ prewet sponge/ 2 pieces of blotting paper/ membrane/ gel/ 2 pieces of blotting paper/ sponge/ black side of the cassette (facing negative electrode). Large gels were transferred at 400mAmps for 4-5 hours in the cold room. Minigels were transferred at 100Volts for 1 hour at room temperature. Gels were stained in Coomassie blue stain to verify transfer. The transferred proteins were visualised by staining the membrane for 5-10 min with Ponceau S (Sigma) for 5-10 min. Excess stain was rinsed off in dH_2O before blocking. When handling nitrocellulose membranes powder-free gloves were worn at all times.

5.8.2. Semi-dry transfer

Semi-dry blotting was performed using a Biometra Fastblot (Biometra Ltd). Gel, nitrocellulose and paper were treated as described above. Two sheets of blotting paper (3MM Whatman paper) were placed on the anode (plate electrode on the body of the Fast Blot), a nitrocellulose sheet was placed on the filter paper, the gel placed on top of the membrane and one layer of blotting paper was placed on top of the polyacrylamide gel. The lid (kathode plate) was connected with the body and gels were transferred at a constant current of 5mAmps per cm^2 for 20-25 min for proteins of 30-50 kDa molecular weight at a gel thickness of 1.0 or 1.5mm. Proteins of 80-120 kDa molecular weight on 0.75mm thick gel were transferred for 30 min. Gels and nitrocellulose membrane were processed as described above.

5.8.4. Immunodetection

Following Ponceau S staining, blots were placed in blocking buffer (Appendix 1) for 1 hour at room temperature or at 4°C over night, in order to block non-specific binding sites. The blocking buffer was decanted and blots were placed in primary antibody. For the initial characterisation of the polyclonal antibodies raised against oligopeptides of caspase 2/ICH-1 serial dilutions of antibody in TTBS plus 5% blotto (Appendix 1) were made to determine the optimal antibody concentration (see below). The incubation period was 1h at room temperature with agitation.

Commercial antibodies were diluted in blocking buffer as suggested in each product description sheet. Anti-CPP32 and Anti-ICH-1 monoclonal antibodies (Transduction Laboratories) were used at a 1:750 dilution in blocking buffer with 1 hour incubation period at room temperature. Anti-PARP monoclonal antibody (Biomol) was used at a 1:1000 dilution in blocking buffer with a 2.5 hour incubation period at room temperature. Alternatively blots were incubated over night at 4°C with all primary antibodies used. Following primary antibody incubation blots were washed 3 x 5 min in TTBS and incubated with the appropriate secondary antibody either swine-anti-rabbit or rabbit anti-mouse horse radish peroxidase coupled (Dako) at 1:1000 dilution in blocking buffer for 1 hour at room temperature or over night at 4°C. The antibody was decanted and blots were washed for 30 min in TTBS with agitation and changes of wash buffer every 10 min. Immunodetection was by enhanced chemi luminescense (ECL) provided in kit form and was carried out according to the manufacture's instruction (Amersham). For miniblots a total 4ml of substrate solution was used, for large blots 8ml were used. The blots were placed on autoradiography film (Hyperfilm-ECL, Amersham) in an autoradiography cassette and exposed for 15 sec up to 1 hour, depending on the signal strength as determined by the amount of target protein present on the membrane. Films were developed in a Hyperprocessor (Amersham).
Following the initial characrterisation of the polyclonal antibodies, Western blotting with all polyclonal antibodies was carried out according to the following protocols (see below).

Western blotting protocol for polyclonal anti-caspase 2 antibody:

- block nitrocellulose membrane 1 hour at room temperature in 10% blotto 0.1% TTBS (or alternatively at 4°C over night)
- incubate membrane 2 hours in primary antibody 1:300 dilution (anti-fragment 1 and 2 antibodies) or 1:3000 dilution (J. Yuan) in 5% blotto 0.1% TTBS
- wash membrane 3x 10 min in 0.1% TTBS
- incubate membrane for for 1 hour (anti-fragment 1 and 2 antibodies) or 30 min (J. Yuan) in secondary donkey anti-rabbit HRP coupled antibody at 1:10 000 dilution in 5% blotto 0.1% TTBS (Pierce)
- wash membrane 3x 10 min in 0.1% TTBS, followed by ECL detection.

Western blotting protocol for polyclonal anti-caspase 2 antibody:

- block nitrocellulose membrane 1 hour at room temperature in 5% blotto 0.1% TTBS
- wash membrane in 0.1% TTBS 2x 5 min
- incubate membrane for 1 hour in primary antibody 1:2000 dilution in 5% blotto 0.05% TTBS (D.W. Nicholson)
- wash membrane 3x 5 min in 0.1% TTBS
- incubate membrane for 1 hour in secondary donkey anti-rabbit HRP coupled antibody at 1:10 000 dilution in 1% blotto 0.05% TTBS (Pierce)
- wash membrane 3x 5 min in 0.3% TTBS
- wash membrane 3x 5min in 0.1% TTBS, followed by ECL detection.

5.8.6. Antibody titrations

To determine the optimal concentration of polyclonal antibodies raised against caspase 2 oligopeptides, 500μg of HeLa protein extract were run on 12.5% SDS using an analytical comb without separate wells. Transfer and blocking was carried out as described above. Miniblots were transferred to a 15 or 21 well multiblotter (Bio-Rad) , wells were rinsed out with TTBS, 1ml of each antibody dilution was made up and 750μl pipetted into separate wells and incubated for 1 hour. Primary antibody dilutions were removed by tilting the multiblotter over and letting the solution run out. Wells were washed separately with three changes of wash buffer, the multiblotter was disassembled and the blot was washed 3x 5 min in changes of wash buffer. Immunodetection was continued for the whole blot as described above.

5.8.7. HPLC and N-terminal amino acid sequencing of recombinant polypeptides

The final purification of the three recombinant proteins by HPLC and amino acid sequencing was carried out in collaboration with the Welmet Protein Characterisation Facility, University of Edinburgh. Recombinant caspase 2 oligopeptides were purified by Reverse-phase HPLC according to Hayes *et al*, 1989. Fractions form the μBondapak C_{18} HPLC column were checked for purity before being sequenced by using an Applied Biosystems 130A Microbore Separation System fitted with an Aquapore RP-300 column (7μm particle size; 2.1mm x 30mm) (Applied Biosystems), that was developed with a linear 8-80% (v/v) gradient of acetonitrile in

Biosystems), that was developed with a linear 8-80% (v/v) gradient of acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid and monitored at 220nm. The polypeptides were subjected to automated sequencing on an Applied Biosystems 477A instrument with a 120A on-line phenylthiohydantoin analyser. Polybrene (2mg) was loaded on to glass-fibre filter disc, which was then precycled three times before being loaded with approximately 1.5nmol of the recombinant polypeptide. After Edman degradation the anilinothiazoline derivatives cleaved from the polypeptide were converted automatically into more stable phenylthiohadantoin forms and separated on an Applied Biosystems PTH C_{18} (5μm particle size; 2.1mm x 220mm) column that was developed with a 0-100% (v/v) linear gradient of acetonitrile. The gradient was formed by using an aq. 5% (v/v) solution of tetrahydrofuran as solvent A and acetonitrile as solvent B. Chromatograghy was performed at 55°C and the column was monitored at 269nm.

5.8.8. Mass spectrometry of recombinant polypeptides

The mass spectrum of the three recombinant caspase 2 oligopepetides was obtained by Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF) using a Voyager Biospectrometry workstation (PerSeptive Biosystems) calibrated with cytochrome c (12360.5 Dalton). Approximately 1μg of recombinant polypeptide in 1μL elution buffer was embedded in 1μl of liquid matrix consisting of small highly absorbing molecules (SIM (4,5 Dimethoxy-4 hydroxycinnamic acid) and αCyano (α-Cyan-4 Hydroxy cinnamic acid) at 10 mg/ml in dH_2O). Matrix embedding was required for absorption of energy from the laser light and isolation of biopolymers from each other. 1μL of the sample mix was then applied to a stainless steel sample probe, dried at room temperature for about 15 min and loaded into the Mass spectrophotometer for analysis. Energy transfer to the sample was at a laser wavelength of 337nm with a typical laser width/duration in the 1-100ns range at 26000 extrcation voltage in a 1.2m flight tube. The transient signal produced was viewed directly on an analog oscilloscope and stored in a transient two channel digital oscilloscope (bandwith >100Mhz). The digitised data generated from successive laser shots was summed, resulting in a TOF mass spectrum.

5.9. RAISING OF RABBIT POLYCLONAL ANTISERA

5.9.1. Antigen preparation

For the first and second immunisation 750μg of each recombinant caspase 2 polypeptide in column elution buffer (Appendix 1) was made up to a volume of 750μl with PBS. This was added to 1.75ml of imject alumn (Pierce) to a total volume of 2.5ml, according to the manufacture's instructions. For the third immunisation (one rabbit/fragment only) 450μg of each column purified large subunit fragment was run in three lanes (~150μg/lane) on a 12.5% SDS-PAGE and transferred to nitrocellulose as described above. Nitrocellulose was stained with Ponceau Red (Sigma) and the band corresponding in molecular weight to the recombinant polypeptide was cut out, destained in dH_2O in a microfuge tube and dried in a 37°C incubator for 10 min. The three nitrocellulose strips were dissolved in 300μl dimethyl ethylsulphoxide (DMSO) to which 300μl PBS was added dropwise under vortexing on a benchtop vortexer.

5.9.2. Immunisation scheme

Four and two weeks prior to immunisation six female black and white Dutch rabbits were prebleed. 15ml of blood was taken at each point. Two rabbits per recombinant caspase 2 polypeptide were immunized with 2ml of the immunogen-alumn mix, injected at two sites (1ml each) subcutaneously near the back of the neck. 10ml of testbleed was taken every two weeks. Eight weeks after the first immunisation all prebleeds and testbleeds were analysed by Western blotting for antibody expression. Rabbits were reimmunised as before and 10ml testbleeds were taken every two weeks for six weeks after the second immunisation. Nine month after the first immunisation for fragment 1 and 2, six month for fragment 3, one rabbit out of the two previously immunized which had previously shown a strong immune response to the recombinant polypeptide (rabbits 68AB, BX08 and 32AB) were reinjected subcutaneously at one site with 600μl antigen solution and 10ml testbleeds were obtained four weeks later.

5.9.3. Purification of antisera

After collection pre- and testbleeds were allowed to clot at room temperature for 30-60 min and were then kept at 4°C over night to allow the formed blood clot to contract. The following day samples were spun at 10000g at 4°C for 10 min in a

Sorvall RT600D centrifuge. Clear serum was taken off with a Pasteur pipette, transferred into 15ml narrow mouth bottles (Nalgene) and stored at -20°C. Immunoglobulin G fractions were isolated from total serum by Protein A or B Sepharose Fast Flow chromatography (Pharmacia Biotech). The column was packed according to the manufacturer's instructions and equilibrated with 20-30ml 20mM phosphate buffer (Appendix 1). About 10ml of serum was applied to the column and allowed to run through. To remove unbound protein the column was washed with 40-50ml 20mM phosphate buffer and the bound IgG fraction was eluted with approximately 10 ml of 0.1M glycine pH 3.0 (Appendix 1). Ten eluate fractions of ~1ml were collected in microfuge tubes to which 50-100µl of 1M Tris-HCL, pH 9.0 had been added to neutralise the eluted fractions. The column was regenerated according to the manufacturer's instructions. Columns were stored at 4°C in 10ml phosphate buffer containing 1% sodium azide. The amount of protein present in each fraction was visualised by pipetting 5µL of each fraction onto dry nitrocellulose, incubating at 37°C for 5 min, staining in rapid Coomassie staining solution (Appendix 1) for ~5 sec, destained in destaining solution (Appendix 1) for 1 min. The nitrocellulose membrane was dried at 37°C for 5 min and fractions containing protein (visible as blue dots) were pooled and the pH was adjusted to 6.5-7.5 with 1M Tris buffer. The amount of protein was quantitated by a Bio-Rad protein assay (see above) and antibody solutions were diluted to 1mg/ml in TBS, pH 7.5 (Appendix 1) and stored as 500µl aliquots at -20°C.

5.10. STANDARD IMMUNOLOGICAL TECHNIQUES

5.10.1. Preparation of cytospins

Cytospins were prepared for immunocytochemistry from three human cell lines Jurkat T cells, H9 cells and HeLa cells. Cells were counted on a haemocytometer (improved Neubauer counting chamber) as described above and diluted in PBS to a concentration of 3-5 x 10^5 cells/ml. Cytospin chambers were assembled and inserted into the cytospin 2 rotor. 100µL of the cell suspension was pipetted into each chamber and cytospin chambers were spun for 3 min at 400g in a Cytospin 2 Shandon centrifuge. The cytospin chambers were dissassembled and the cytospins were air dried for 5 min, fixed in acetone for 5 min and air dried before storage. Cytospins for

immediate use were stored at 4°C, for long term storage cytospins were kept at -20°C.

5.10.2. Preparation of frozen and parraffin embedded tissues

Human tissues were collected at the time of operation for treatment of the diagnosed disease. In addition human organs were collected from routine autopsy, where the autopsy was performed less than 12 hours after death to establish cause of death. Tissues were fixed in buffered formalin for 16 hours and then processed to paraffin wax in VIP (vacuum impregnating processor). Paraffin embedded sections were cut on a mycrotom to a thickness of 3μm.

5.10.3. Antigen retrieval by microwaving or trypsinising

Antigen retrieval of certain antigens, in order to reverse cross-linking caused by paraffin embedding, was carried out by trypsinisation or microwaving of paraffin sections. In the same way as untreated sections, sections for microwaving and trysinisation were deparrafinised by incubation in xylene (Genta Medical) for 5-10 min and rehydrated by passing through descending grades of alcohol to H_2O (from adsolute ethanol, to 75%, to 65%, to tap H_2O). Endogenous peroxidase activty was blocked by incubating sections for 20 min in 1% hydrogen peroxide solution (Sigma) (Appendix 1) followed by a 5 min wash in running water. Sections were then incubated for 20 min at 37°C in 0.1% trypsin solution (Appendix 1) followed by a 5 min wash in running water and a rinse in TBS for 5 min. Alternatively section were transferred to a microwavable container, immersed in excess citrate buffer (Appendix 1), covered to prevent evaporation, microwaved 3x 5 min at 700Watts, allowed to cool in buffer for 20 min, followed followed by a 5 min wash in running water and a rinse in TBS for 5 min. Sections were then used for immunohistochemical analysis.

5.10.4. Immunohistochemistry and immunocytochemistry

All immunohisto- and immunocytochemical staining was carried out in semi-automated 'Sequenza' (Shandon) wet chamber, which allows staining of up to 50 slides simultaneously. Slides of tissue sections or cytospins were assembled with cover plates submerged in TBS to stop creation of air bubbles and were transferred into the sequenza rack. Assembled sections were preincubated with 100μl of either 20% normal rabbit serum in TBS (Sapu) or 20% normal swine serum in TBS (SeroTech)

(depending on the species in which the secondary antibody was raised) to block non-specific secondary antibody binding sites. After 10 min preincubation 100µl optimally diluted primary antibody (diluted in 20% normal serum) was added to each well and sections were incubated for 30 min at room temperature or over night at 4°C. Primary antibody was washed off in two changes in 5 min intervals of TBS (sequenza well filled twice). 100µL of either swine anti-rabbit or rabbit anti-mouse horse radish peroxidase (HRP) coupled secondary antibody (Dako) diluted 1:100 in normal serum was then added to each well and incubated for 30 min, followed by two washes with TBS as above.

Alternatively sections were incubated with a rabbit anti-mouse unlabelled secondary antibody (Dako Z259) diluted 1:5 for 30 min, followed by two washes in TBS as above. Sections were then treated with a secondary mouse alkaline phosphatase-anti alkaline phosphatase monoclonal antibody (APAAP) (Dako D651) diluted 1:20 in normal rabbit serum for 30 min, followed by two washes with TBS as above.

5.10.5. Visualisation with DAB

Immunecomplexes of tissue sections or cytospins treated with a HRP coupled secondary antibody were visualised by incubation for 3-5 min with 100µl 3,3-diaminobenzidine (DAB) (Sigma) substrate solution (Appendix 1). Slides were washed in two changes in 5 min intervals of TBS (sequenza chambers filled twice), followed by disassembly of the sequenza chambers and further 5 min wash under running water. Sections were counter stained for ~45 sec in Harris haematoxylin (Shandon), washed under running water, stained in Scot's tap water substitute for ~45 sec, washed under running tap water, dehydrated by 1min incubation in ascending grades of ethanol bath (from H_2O, 65% ethanol, 75% ethanol to absolute ethanol) and transferred into xylene. Sections or cytospins were then mounted in the fume hood with pertex mounting medium (CellPath).

5.10.6. Fast Red visualisation

Immunecomplexes of tissue sections treated with an APAAP coupled secondary antibody were visualised by incubation for 30 min in the dark with 500µl Fast Red substrate solution (Appendix 1). Sections were washed and counter stained

as described for DAB visualisation but were transferred into water and mounted with aquamount (BDH).

5.10.7. ELISA

Serial dilutions of subunits of four recombinant caspase as well as the three recombinant caspase 2 oligopeptides were made up in ELISA coating buffer (Appendix 1) and 100µl of each solution was added to separate wells of a 94 well disposable ELISA microtiter plate (Corning). On each plate blanks wells coated with coating buffer only followed by incubation with secondary antibody were included. Plates were either coated for 3 hours at room temperature or were left over night at 4°C and the coating buffer was removed by shaking the plate out. Primary antibody was diluted in antibody dilution buffer (Appendix 1). 100µl of appropriately diluted primary antibody was added to each well and incubated at room temperature for 2-3 hours. Antibody solution was removed and wells were automatically washed 3x in ELISA wash buffer (Appendix 1) in a multi reagent washer (Dynatech MRW). Swine anti-rabbit HRP coupled secondary antibody was diluted 1:1000 in dilution buffer. 100µl of the secondary antibody dilution was added to each well, incubated at room temperature for 2-3 hours and plates were washed on the automated plate washer as above. 100µL of either ODP (O-Phenylenediamine dihydrochloride, Sigma) substrate buffer (Appendix 1), producing a soluble end product that is yellow in colour or TMD (Tetramethylbenzidine free base) substrate buffer (Appendix 1), producing a soluble end product that is blue in colour was added to each well with a multipipetter to ensure equal incubation time and was incubated for 20-30 min. The reaction was stopped by addition of 50µl of 3N HCL (for ODP buffer), resulting in an orange colour reaction or 50µl of 2M H_2SO_4 (for TMD buffer), resulting in a yellow solution to each well. Absorbance was measured on a MR 5000 ELISA plate reader (Dynatech) at 490nm (for ODP buffer) or 450nm (for TMD buffer) and the data was analysed using BioLynx, Version 2.20 computer software (Dynatech Laboratories).

5.11. GENRAL CELL CULTURE TECHNIQUES

5.11.1. Routine culture maintenance

Jurkat, HeLa, H9, Priess, Panc1, Mia and CF Pac cells were cultured in RPMI medium supplemented with 10% foetal calf serum, glycine 0.1mM, 1 unit/ml penicillin

(GiBco BRL) and 100μg/ml streptomycin (GiBco BRL). Cultures were kept at a temperature of 37°C, in a humidified atmosphere of 5% CO_2, 95% air and were maintained in exponentially growing phase. Cells were passaged routinely at a 1:10 dilution, when about 75% confluence was reached, which provided about 7.5 x 10^6 cells from a 175cm^2 flask. To subculture cells, semiadherent Priess cells were disaggregated by a sharp tap to the flask and the cell suspension, like other nonadherent cells was transferred to Falcon tubes and centrifuged for 5min at 1000rpm in a bench top centrifuge (MSE), and then resupended in fresh medium. Cultures of adherent cells were passaged, usually about 1:6-1:10, just before confluence was reached. The medium was aspirated and the cells were washed once with PBS. The PBS was aspirated and 5ml of trypsin for a 100cm^2 flask was added, dispersed evenly and the cells were incubated at 37°C for 3-5 min. As soon as the cells started to detach they were dissociated by vigourous knocking of the side of the flask with the palm of the hand. After checking the dissociation under the microscope, 10ml of medium was added and 1ml transferred to a fresh flask with 30ml of complete medium.

5.11.2. Freezing

Cells when not required were stored in the vapour over liquid nitrogen. To freeze down vials, a 175cm^2 flask was grown to near confluence, trypsinised as described above (adherent cells), pelleted by centrifugation for 5min at 1000rpm, washed once in sterile PBS, respun and then resuspended in 10ml of cold medium containing 10% DMSO and 40% foetal calf serum (freezing mix) and 1ml transferred to a cryotube (Nunc). Cells were then promptly transferred to a -80°C freezer overnight at an approximate cooling rate of 1°C/min, and moved to storage over liquid nitrogen the following day.

5.11.3. Thawing

To bring frozen cells back to culture, a freezing vial was removed from liquid nitrogen and thawed rapidly by the addition of 1ml pre-warmed medium. The cells were washed in 10-20ml RPMI medium before replating.

5.12. INDUCTION OF APOPTOSIS IN HUMAN CELL LINES

5.12.1. Treatment with etoposide

Human Jurkat T cells and Priess B cells were induced to undergo apoptosis by incubation with 50μM etoposide (in DMSO) added directly to the tissue culture medium. Control cells were incubated with the equivalent amount of DMSO only. At regular intervals of 10-12 hour time course, samples of cells were fixed to assess apoptotic morphology (see below) and cell extracts were prepared for Western blotting analysis (see above).

5.12.2. Treatment with anti-Fas antibody

For induction of apoptosis by anti-Fas treatment human Jurkat and Priess cells in logarithmic growth phase were counted in a haemocytometer (improved Neubauer counting chamber). The required number of cells (2×10^7 cells/time point) were centrifuged at 1000rpm for 5 min in a bench top centrifuge (MSE). The culture medium was taken off, pelleted cells were washed in 15ml of sterile PBS, centrifuged as above and put on ice. Cells were incubated on ice for 30 min with 200ng of anti-Fas monoclonal antibody (clone CH11; Immunotech) per 1×10^6 cells. Antibody and cells were mixed by gentle pipetting. Control cells were incubated on ice for 30 min without the addition of anti-Fas antibody. Following incubation on ice cells were resuspended in medium (20ml of medium/2×10^7 cells) and at regular intervals of a 10-12 hour time course, samples of cells were fixed to assess apoptotic morphology (see below) and cell extracts were prepared for Western blotting analysis (see above).

5.12.3. Treatment with staurosporine

Human Jurkat T cells and Priess B cells were induced to undergo apoptosis by incubation with 1μM staurosporine (in DMSO) added directly to the tissue culture medium. Control cells were incubated with the equivalent amount of DMSO only. At regular intervals of 10-12 hour time course, samples of cells were fixed to assess apoptotic morphology (see below) and cell extracts were prepared for Western blotting analysis (see above).

5.13. EVALUATION AND QUANTIFICATION OF APOPTOSIS

5.13.1. Fluorescence microscopy

Cells for morphological analysis (1 x 10^6) were washed once in PBS, centrifuged for 2 min at 6500rpm, resuspended in 50µl of PBS, fixed by the addition of 50µl of 4% formaldehyde in PBS and stored at 4°C. 5µl of fixed cells were mixed with 5µl of acridine orange (10µg/ml in PBS) and a minimum of 500 cells were scored under UV light on a Zeiss Axiphot fluorescent microscope for apoptotic morphology.

5.13.2. Transmission electron microscopy

Tissue cultured cells were washed once in PBS, centrifuged for 2 min at 6500rpm, resuspended in 50µl of PBS, fixed by the addition of 50µl of 4% formaldehyde in PBS and stored at 4°C until processed for electron microscopy. The suspension of formaldehyde fixed cells was centrifuged for 2 min at 2000rpm and resuspended and refixed in 3% glutaraldehyde containing 0.1M sodium cacodylate buffer at 4°C for 30 min. Samples were centrifuged as above and washed in 0.1M sodium cacodylate buffer at 4°C for 15 min. Following centrifugation as above samples were post fixed in 1% osmium teroxide containing 0.1M sodium cacodylate buffer at 4°C for 30 min. Samples were then dehydrated through various grades of ethanol (from 10%, 50%, 75% to 100% ethanol), 3x 10 min incubation per grade up with three changes of ethanol and centrifugation of samples was carried out as before. Dehydrated samples were transferred into solvent resistant capsules and incubated in two changes (10 min each) of propylene oxide, centrifuged in between steps. Samples were impregnated in polyethylene capsule in warm araldite epoxy resin (enough resin just to cover the sample) at 56°C overnight. The capsules were filled up with fresh epoxy resin the following morning and incubated for an additional 2-3 days at 56°C. Sections of approximately 50-60nm were cut on an ultramicrotome (LKB Nova), stained with uranyl acetate and lead citrate and analysed on a Philips CM12 Transmission electron microscope

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7.0 APPENDIX 1

PBS
9g NaCl
0.262g monobasic sodium phosphate monohydrate (FW 138)
1.15g dibasic sodium phosphate (FW 142)
made up to 1 litre with ddH_2O, pH 7.4, autoclaved
alternatively:
1 PBS tablet (Oxoid) dissolved in 100ml ddH_2O, autoclaved

Tween PBS
PBS with 0.1% Tween-20

dNTP stock solution
10µl of each 100mM stock dNTP
made up to 100µl with ddH_2O
stored in aliquots at -20°C

6x gel loading buffer
30% glycerol
0.25% xylene cyanol FF (Bio-Rad)
0.25% bromophenol blue (Fisher Scientific) in ddH_2O

10x TBE
48.4g Tris
55g boric acid
40ml of 0.5M EDTA
made up to 1 litre with ddH_2O, pH 8.0

TE
10mM Tris
1mM EDTA
in ddH_2O

S.O.C. medium
2g Bacto-tryptone
0.5g yeast extract
NaCl, $MgCl_2$, $MgSO_4$ (10mM each)
KCl (2.5mM)
glucose 20 mM
made up to 100ml with dH2O, filter sterilised (0.2µ filter)

LB medium
10g Bacto-tryptone
5g yeast extract
10g NaCl (0.17mM)
made up to 1 litre with dH_2O, pH 7.5, autoclaved

LB agar
500 ml LB medium
7.5g (1.2% w/v) bacto-agar
autoclaved, cooled to 50°C, supplemented with antibiotic (100µg/ml ampicillin; 34µg/ml chloramphenicol)

4x Ni^{2+} affinity column elution buffer
4M imidazole
2M NaCl
80mM Tris-HCL pH 7.9
diluted to 1x buffer with ddH_2O prior to use, 6M urea added if used for elution of insoluble recombinant proteins

6% denaturing polyacrylamide gel
75ml of 19:1 acrylamide-bisacrylamide stock (EasyGel)
250g urea
50ml of 10x TBE
175ml dH_2O
stored at 4°C, stable for up to ~ 4 weeks
add 100µl TEMED and 100µl ammonium persuphate to 100ml gel solution

IPTG stock solution
2.38g IPTG in 100ml ddH_2O (100mM), stored in aliquots at -20°C

4x running gel buffer
1.5M Tris-HCl (36.3g; FW 121.1)
add 150ml ddH_2O, adjust pH to pH 8.8 with HCL, ddH_2O to 200ml, stored up to 3 month at 4°C in the dark

4x stacking gel buffer
0.5M Tris-HCl (3.0g; FW 121.1)
add 40ml of ddH_2O, adjust to pH 6.8 with HCl, ddH_2O to 50 ml, stored up to 3 month at 4°C in the dark

running gel overlay
25ml running gel buffer
1.0 ml 10% SDS
ddH_2O to 100ml, stored at 4°C up to 3 month in the dark

2x SDS sample buffer
0.125M Tris-HCl, pH 6.8 (2.5ml stacking gel buffer)
4% SDS (4.0ml 10% SDS)
20% v/v glycerol (2.0ml glycerol)
0.2M dithiothreitol (0.31g DTT; FW 154.2)
0.02% bromophenol blue (2mg)
ddH_2O to 10ml, stored in 500µl aliquots at -20°C for up to 6 month

rapid stain fixing solution
25% isopropanol (250ml)
10% acetic acid (100ml)
made up to 1 litre with dH_2O

rapid Coomassie blue stain
0.06% Coomassie blue G-250 (0.6mg)
10% acetic acid (100ml)
made up to 1 litre with dH_2O

destaining solution
7% actic acid (70ml)
5% methanol (50ml)
made up to 1 litre with ddH_2O, stored indefenitely at room temperature

sodium phospate lysis buffer
0.1M $NaPO_4$
0.05% Nonidet P-40
adjust to pH 6.9
prior to use add 2.5mg/ml Pefabloc protease inhibitor cocktail (Boehringer Mannheim)

cell lysis buffer
62.5mM Tris-HCl (12.5ml 4x stacking gel buffer, pH 6.8)
2% SDS (20.0ml 10% SDS)
6M urea (36g urea; FW 60.06)
10% glycerol (10.0ml)
0.1M DDT (1.55g; FW 154.2)
made up to 100ml with ddH_2O, aliquoted into 5ml
stored for up to 6 month at -20°C
prior to use add protease inhibitors at 1:1000 dilution: PMSF (100mM stock in absolute ethanol), protease inhibitor cocktail (2.1M leupeptin; 1.5M pepstatin A; 1.7M antipain; 1.6M chymostatin, made up in DMSO, stored at -20°C in 10µl aliquots)

HEPES lysis buffer (pH7.0)
100mM HEPES (FW 238.3)
1% TritonX-100
in ddH_2O
add protease inhibitors at 1:1000 diltution prior to use: PMSF (100mMM), leupeptin (2.1M), antipain (1.7M), pepstatin A (1.5M) and chymostatin (1.6M)

Towbin transfer buffer (pH 8.2-8.4)
25mM Tris (30.3g, FW 121.1)
192mM glycine (144.1g; FW 75.07)
10g SDS
make up to 8 litres with ddH_2O
add 2 litres of methanol

5x TTBS
100mM Tris-HCl (302,75g; FW 121.1)
add 4 litres of ddH_2O
adjust pH to 7.5 with concentrated HCL
225g NaCl
0.1% Tween-20 (25ml)
made up to 5 litres with ddH_2O, stored at 4°C

TBS
as TTBS but without the Tween-20

blocking buffer (Western blotting)
1x TTBS plus 5% or 10% blotto (Marvel, dried milk powder)

stipping buffer (Western blotting)
0.1M glycine pH 2.9

20 mM phosphate buffer (pH 7.3)
stock A: 0.4M sodium di-hydrogen phosphate (6.2g) ($NaH_2PO_4H_2O$; FW 156.01) to 100ml with ddH_2O
stock B: 0.4M di-sodium hydrogen phosphate (7.09g) ($Na_2H_2PO_4H_2O$; FW 178) to 100ml with ddH_2O
take 5.6ml of stock A, add 14.4 ml of stock B, make up to 350ml with ddH_2O, adjust pH to 7.3, make up to 400ml with ddH_2O

0.1M glycine pH 3.0
500ml of 0.2M glycine (15.01g made up to 1 litre with ddH_2O; FW 75.07)
pH adjusted with concentrated HCl to pH 3.0
made up to 1 litre with ddH_2O

hydrogen peroxide blocking solution
3ml hydrogen peroxide (30% w/w stock, Sigma)
97ml ddH_2O

trypsin solution (antigen retrieval)
0.1% trypsin (1mg) (ICN Biomedicals Inc.) dissolved in 100ml of 0.1% CaCl adjusted to pH 7.6 with 0.2M Tris

excess citrate buffer
10mM citric acid (1.05g monohydrate, FW 210.1)
made up to 450ml with ddH_2O
pH adjusted to pH 6.0 with 2N NaOH
made up to 500ml with ddH_2O

DAB substrate solution
made up fresh prior to use
use 4.8ml ofDAB buffer:
24ml 0.2M Tris
38ml 0.1M HCL
0.0681g imidazole
38ml ddH_2O
pH adjusted to pH 7.6
add 100µl DAB substrate
100µl frozen aliquots at 25mg/ml (50x stock)
add 100µl of 1% hydrogen peroxide:
10µl hydrogen peroxide (30% w/w stock, Sigma) in 290µl ddH_2O

Fast Red substrate solution
2mg Naphthol AS MX phosphate dissolved in 200µl N,N Dimethylformamide, made up to 10ml with 0.1M Tris buffer pH 8.0, add 10µl of 1M Levamisole (to block enogenous alkaline phosphatase), prior to use 40mg Fast Red (Sigma) added and solution filtered through Whatman filter paper to remove undesolved material

ELISA coating buffer
dissolve one carbonate/bicarbonate buffer tablet (Sigma C3041) in 100ml , followed by two washes with TBS as above.

ELISA antibody dilution buffer
PBS with 1% BSA (FirstLink (UK) Ltd.)

ELISA wash buffer
0.1% BSA
0.05% Tween 20
in PBS

ODP substrate buffer
make up fresh prior to use
take 10ml of:
25.7ml 0.2M Na_2PO_4
24.3ml 0.1M citric acid
made up to 100ml with ddH_2O
pH adjusted to 5.0
add ODP substrate tablet (Sigma)
add 4µl 30% w/v H_2O_2

trypsin solution (tissue culture)
0.1% trypsin in sterile PBS

8.0 APPENDIX 2

Figure 1. pET vector diagram. Shown is the map for the pET23a(+) vector which is identical to pET23d except of the following criteria: pET23d is a 3663bp plasmid; the BamH1 site is in the same reading frame as in pET23c containing a C-terminal histidine tag (six histidines); an Nco1 site is substituted for the Nde1 site with a net 1bp deletion at position 238, as a result Nco 1 cuts pET23d at 234, and Nhe1 cuts at 229 (adapted from the pET System Manual, Novagen).

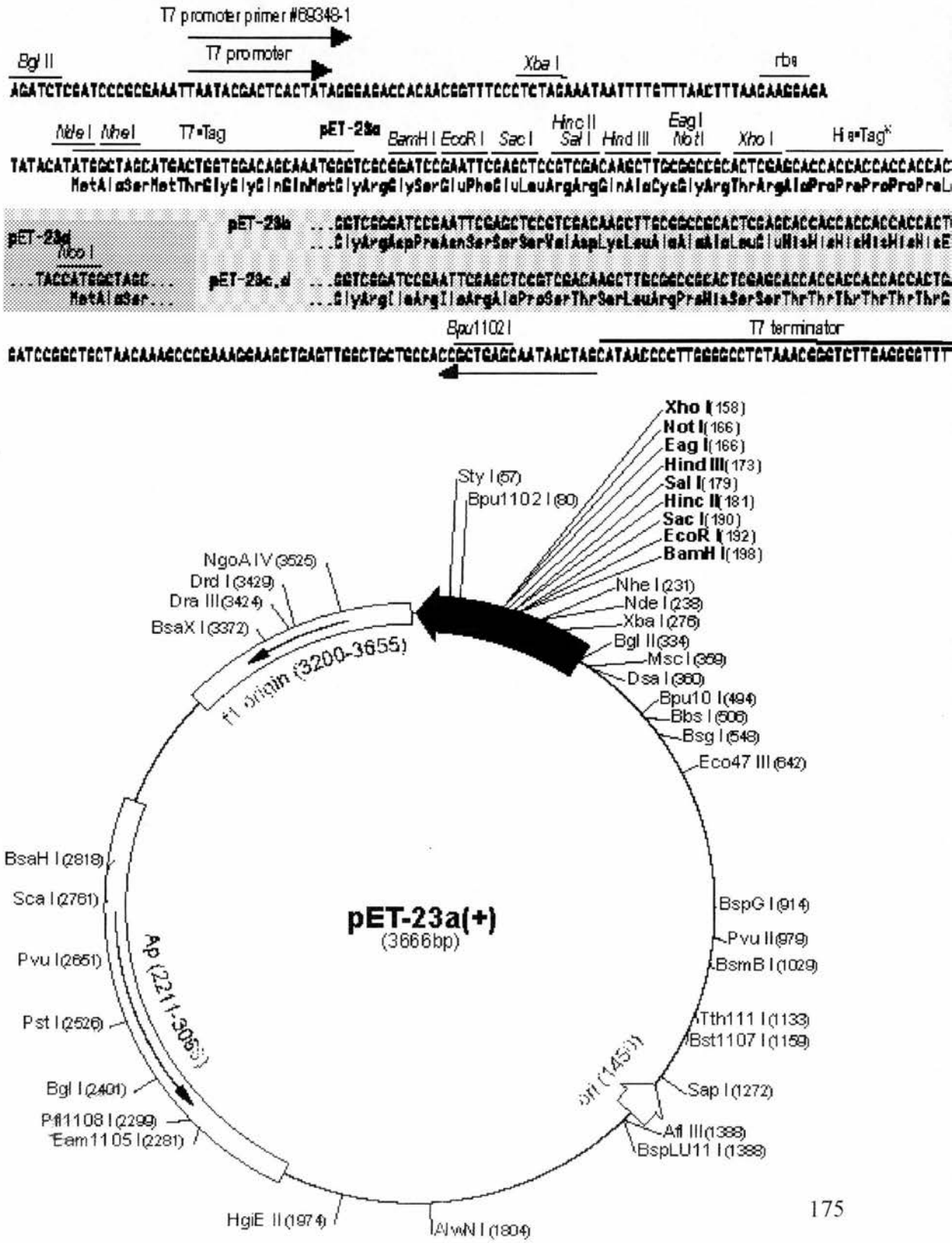


Figure 2. Control elements of the pET system. Caspase 2 polyucleotide sequences cloned into the bacterial expression vector pET23d1-3. Shown is the induction of *caspase 2* expression by addition of IPTG to the *E.coli* BL21pLysS culture (adapted from Novagen technical bulletin).

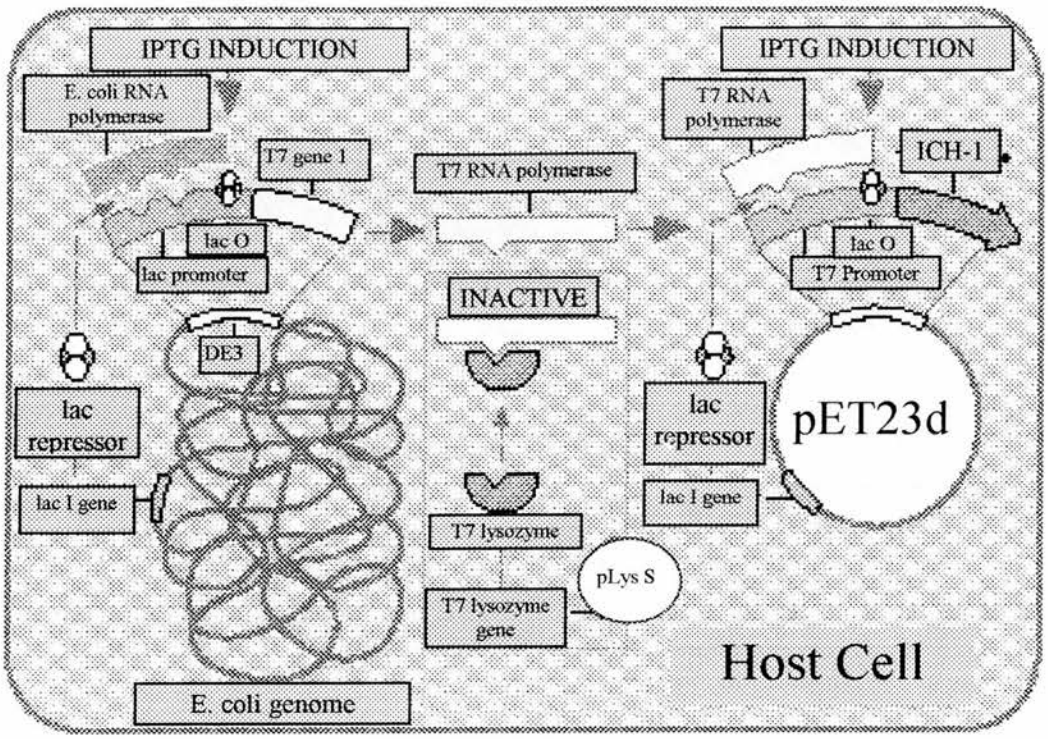


Figure 3. Antigen for caspase 2 polyclonal and monoclonal antibodies. Structure of procaspase 2 with marked subunits (p18 and p12) and prodomain and linker region (black boxes). Immunogen of commercial anti-caspase 2 antibodies depicted in relation to procaspase 2. **A** Immunogen for the Transduction Laboratories anti-caspase 2 antibody, mouse monoclonal IgG1 antibody, raised against 19.5kDa protein fragment (176 amino acids) corresponding to amino acids 225-401, including parts of the large subunit , the small subunit and the linker region of procaspase 2. **B** Santa Cruz anti-caspase 2 (ICH-1 $_{S/L}$) antibody, rabbit polyclonal IgG, raised against a peptide corresponding to amino acids 2-18 mapping at the amino terminus of the procaspase 2.

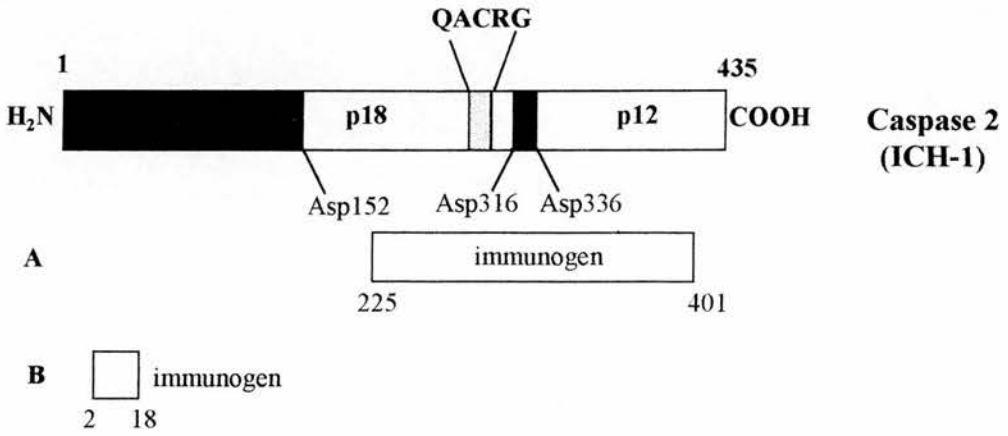
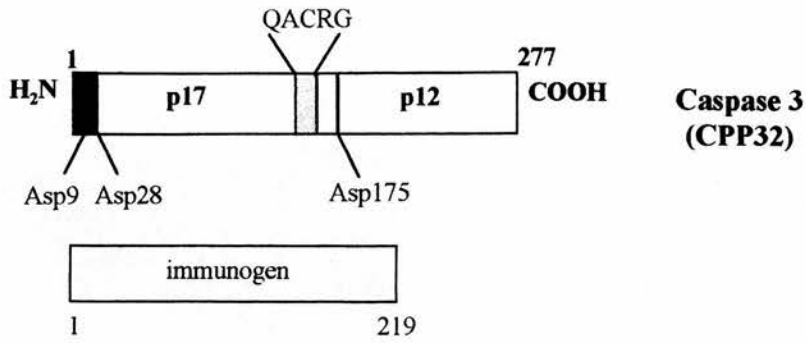


Figure 4. Antigen for caspase 3 monoclonal antibody (Transduction Laboratories). Structure of procaspase 3 with indicated positions of subunits (p17 and p12) and prodomain (black box). IgG1 mouse monoclonal anti-caspase 3 antibody raised against a 24.7kDa protein fragment corresponding to amino acids 1-219 of procaspase 3. Position of the immunogen depicted in relation to procaspase 3.



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ICE-Like Proteases and Cell Death

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Introduction

Apoptosis was first recognized as a distinctive process because of its characteristic structural changes. These affect the nucleus, cytoplasm, cytoskeleton and plasma membrane coordinately and occur regardless of the affected cell type or lineage in response to both physiological and pathological stimuli. The cellular triggers of apoptosis vary widely including activation of molecules in response to particular types of cell damage (eg. p53, ceramide) and ligand binding to specific receptors (eg. Fas, TNFR). Downstream of these triggers lie further regulatory molecules which are important in initiating or forestalling the final common events but do not contribute directly to them, such as the *reaper* gene product in Drosophila and the various members of the *bcl-2*, family. The effector events themselves depend on activation of a set of proteases with a cysteine in their active site and remarkably conserved structure and function between species. These cysteine proteases appear to be directly responsible for the structural changes of apoptosis and are the subjects of this article.

Current appreciation of the significance of cysteine proteases as effectors of apoptosis originated from the identification and cloning of the *C. elegans* cell death gene, *ced-3* (*c*ell *d*eath abnormal). This gene, which is essential for normal programmed cell death in the nematode (*Ellis,* 1986) encodes a protein of 503 amino acids with 29% amino acid identity to the human interleukin-1β (IL-1β) converting enzyme (ICE) (*Yuan,* 1993; *Thornberry,* 1992; *Miura,* 1993; *Xue,* 1996). Structurally unrelated to any other known class of proteases, ICE and Ced-3 are the founder members of a growing new ICE-like protease family. To date known family members include ICH-1/NEDD-2 (*Wang,* 1994; *Kumar,* 1994), prICE (*Lazebnik,* 1994), CPP32/Yama/Apopain (*Fernandes-Alnemri,* 1994; *Tewari,* 1995; *Nicholson,* 1995), TX/ICH-2/ICErelII (*Faucheu,* 1995 *Kamens,* 1995; *Munday,* 1995), ICErelIII/TY (*Munday,* 1995; *Faucheu,* 1996), Mch2 (*Fernandes-Alnemri,* 1995); Mch3/ICE-LAP3 *Fernandes-Alnemri,* 1995; *Duan* 1996), CMH-1 (*Lippke,* 1996), FLICE/MACH (*Muzio,* 1996; *Boldin,* 1996). Here we discuss the common structural features of the ICE-like proteases, their substrates in apoptosis and their regulation in terms of biochemical interactions between individual family members and other components of the apoptotic pathway.

Composition and Structure of ICE-Like Proteases

Structural studies are more complete for ICE than for any of the later discovered family members. ICE was originally purified from the cytosol of the human monocytic cell line THP.1 as a heterodimeric enzyme composed of two subunits of 10 (p10) and 20 (p20) kDa molecular weight (*Thornberry,* 1992). ICE cleaves the inactive 33 kDa IL-1β precursor (proIL-1β) at Asp116-Ala117 to generate the

17 kDa fully active cytokine IL-1β. ICE cDNA has an open reading frame of 1212 nucleotides encoding a proenzyme of 404 amino acids with a predicted molecular weight of 45 kDa. The mature form of ICE is derived from this 45 kDa precursor by proteolytic removal of the N-terminal 119 amino acids and an internal fragment spanning residues 289-316. Cleavage occurs by autocatalysis at Asp-X bonds where X is Ser104, Asn120, Ser298 or Ala317 (*Thornberry,* 1992; *Wilson,* 1994) (Figure 1).

The catalytic mechanism and the substrate specificity of ICE have been revealed by the use of synthetic oligopeptide inhibitors. These small peptides (usually four amino acids) are engineered to mimic the proteolytic substrate cleavage site of the enzyme and contain the amino acid residues essential for catalytic recognition. They are synthesised as peptide aldehydes or chloromethylketones and act as competitive inhibitors that undergo nucleophilic addition of the catalytic cysteine to form thiohemiacetals and thiohemiketals (*Thornberry,* 1995). The synthetic tetrapeptide acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO) inhibits the catalytic mechanism of ICE by binding covalently to the sulphur atom of Cys285 (*Thornberry,* 1992). This type of observation, together with results from site-directed mutagenesis led to the classification of ICE as a cysteine protease and demonstrate that ICE displays substrate specificity for an aspartic acid residue at the P1 position (the first amino acid N-terminal to the cleavage site). Although the catalytic cysteine is located in the p20 subunit at position 285, X-ray crystallographic studies show that the active site is formed by amino acids from both subunits and that catalysis also requires p20 subunit residues His237 and Gly238. P20 amino acids Arg179, Gln283

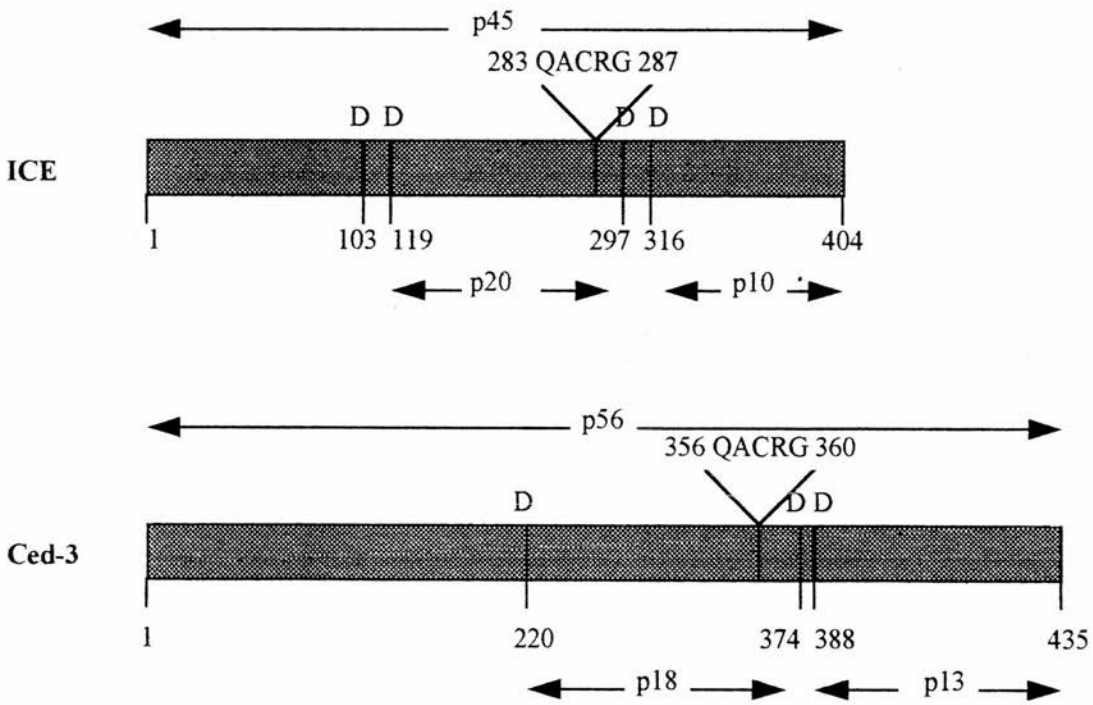

Fig. 1: Structure of ICE and Ced3.

together with p10 residues Arg341 and possibly Ser347 are the critical elements in the formation of the binding pocket for the carboxylate side chain of P1 Asp (*Walker,* 1994; *Wilson,* 1994). From crystallographic studies it has been proposed that two p20/p10 subunits heterodimerize to form the tetrameric active enzyme (*Thornberry,* 1992; *Wilson,* 1994, *Walker,* 1994). Unless large conformational changes accompany autoprocessing, it is probable that the p20 and p10 contributing to each active site derive from different precursor proteins. The p45 precursor protein itself has been shown to form oligomers in transfected COS cells. This oligomerization is a seemingly prerequisite state for autoprocessing to the active enzyme (*Gu,* 1995). Global fold, topology and quaternary structure of ICE is unlike that of proteases of other classes (*Wilson,* 1994; *Walker,* 1994).

ICE and CPP32 (cysteine protease p32) are so far the only family members with known crystal structure. In CPP32 two p17 and p12 subunits heterodimerize, as in ICE, to form the active tetrameric enzyme (*Rotonda,* 1996). Sequence comparisons between the other family members and ICE show approximately 30% amino acid identity and structural analysis suggests that all members are synthesized as inactive proenzymes in the same way as ICE. The residues of ICE important for recognition of P1 Asp and for catalysis are conserved in every member, together with a pentapeptide QACRG, containing the active site cysteine. This suggests that all members share the same catalytic mechanism. There is more diversity, however, in the amino acids that putatively line the groove where the P2-P4 residues of the substrate lie, corresponding in ICE to Val338, Trp340, His342, Pro343, Arg383 and Gln385. These amino acids are most likely responsible for the differing substrate affinities amongst the various family members. Further differences exist between individual family members in the molecular weight of the proenzyme and the two subunits of the active enzymes. The CPP32 proenzyme for example lacks the 2 kDA linker peptide between the two subunits and has a prodomain that is substantially shorter than ICE (*Nicholson,* 1995). For most of the other ICE-like proteases the exact subunit sizes are still undetermined. Molecular masses have been predicted, however, and together with sequence comparison data suggest that ICH-2/TX/ICErelII and ICErelIII/TY are likely to be proteolytically processed to subunits analogous to those of mature ICE. ICH-1/NEDD-2, 2, Mch3/ICE-LAP3 and CMH-1 are in sequence more closely related to Ced-3 and CPP32, have generally smaller molecular weight proenzyme forms and are believed to be processed to subunits equivalent of CPP32. The ICE-like protease family has thus been further subdivided into the ICE-subfamily and the Ced-3 subfamily.

Involvement of ICE-Like Proteases in Apoptosis

Definitive proof that a particular protein contributes to the effector pathway of cell death requires that it is expressed in cells competent to activate the pathway, that overexpression initiates death and that downregulation or specific inhibition block cell death. At present very few of the ICE family proteases fulfill all these requirements. Apoptosis has been induced in a variety of mammalian and insect cells following overexpression of ICE and most of the other members of the ICE-like protease family (*Miura,* 1993). This evidence alone is insufficient proof of a specific role of those ICE-like proteases in cell death, however, as introduction of other known proteases of entirely different specificities has been claimed to induce death with many of the characteristic morphological features of apoptosis including membrane blebbing, chromatin condensation and DNA fragmentation (*Williams,* 1994). Evidence for the

physiological role of ICE-family proteases derives from observation of the efficacy of supposedly specific protease inhibitors (natural or synthetic), the biology of genetically altered ("knock-out") mice, and protease activity in apoptotic cell extracts.

Natural inhibitors

The existence of natural macromolecular viral inhibitors of ICE-like proteases, which also inhibit apoptosis, provides suggestive evidence that apoptosis plays a significant role in cellular anti-viral defence. Viral inhibitors of ICE-like proteases include the cowpox virus *crmA* and the baculovirus *p35* gene.

Induced expression of the cowpox virus gene *crmA*, which encodes a 38 kDa protein of the serpin family, suppresses ICE-activated apoptosis in Rat-1 cells (*Miura*, 1993). *CrmA* expression from a microinjected vector inhibits the death of neurons that follows withdrawal of nerve growth factor (*Gagliardini*, 1994) or isolation from contact with extracellular matrix (*Boudreau*, 1995). Mammalian MCF7 (breast epithelial) and BJAB (B-cell lymphoma) cell lines stably transfected with *crmA* are rendered resistant to TNF and Fas-mediated apoptosis, showing no cytopathic effect even at high doses of the death stimulus (*Tewari*, 1995). Hence the death pathway in all these systems converges on a CrmA-inhibitable step. Successful inhibition of certain ICE-like proteases however, appears to require high levels of expression of the *crmA* gene, not necessarily achieved during viral infection. Studies of enzyme kinetics therefore add important information. CrmA has a 10,000 fold preference for ICE over CPP32. On the basis of the high sequence similarity between CPP32 and Mch3/ICE-LAP3 the latter might not be inhibited by CrmA during cowpox virus infection. CrmA is known to inhibit processing of IL-1β (*Ray*, 1992; *Komiyama*, 1994), a substrate of ICE, and thus these results are compatible with activation of ICE in the final common pathway of apoptosis. They leave unresolved, however, the question which or how many other ICE-like proteases may be involved, since structural similarity between family members would predict that many of these should also be inhibited by CrmA.

P35 blocks apoptosis in baculovirus infected insect cells, resulting in increased virus production (*Crook*, 1993; *Clem*, 1994) and can block cell death in both vertebrate and invertebrate cells. Expression of p35 in *Drosophila melanogaster* abrogates death induced by *reaper* overexpression (*White*, 1996). P35 expression in *C. elegans* and in *Drosophila melanogaster* embryos also prevents their physiological programmed cell death (*Sugimoto*, 1994; *Hay*, 1994) although *crmA* expression has no antiapopotic effect. In mammalian cells overexpression of p35 blocks proteolytic activity of ICE and purified p35 inhibits at least three other family members (ICH-1, ICH-2 and CPP32) (*Bump*, 1995). It is thus possible that p35 has a broader specificity than CrmA. Interestingly, both p35 and CrmA possess internal cleavage sites for ICE-like proteases. Cleavage at these sites results in a conformational change rendering the inhibitor less easily dissociable from the enzyme and thereby prolonging its inhibitory effect. In elegant experiments, the ability to arrest programmed cell death in *C. elegans* embryos was conferred upon CrmA by exchanging its cleavage site for that of p35, suggesting that cell specificity of these endogenous inhibitors may be mediated by very subtle molecular changes (*Xue*, 1995).

Synthetic inhibitors

Synthetic peptides which contain the cleavage site for ICE in proIL-1β are suitable substrates for the enzyme. Such peptides competitively inhibit the cleavage of natural substrate within cells and cell extracts, particularly if presented as covalently binding aldehyde or

chloromethylketone derivatives. A direct copy of the cleavage site in proIL-1β (at Asp27 and Asp116) is Tyr-Val-His-Asp (YVHD), but broad substitution in the P2 position is tolerated and in practice YVAD is a better inhibitor of ICE (*Thornberry*, 1992). YVAD coupled to a DEAE column has been used to purify active ICE to homogeneity from cytosolic extracts, demonstrating the avidity and specificity of the peptide-enzyme binding (*Thornberry,* 1992). It effectively inhibits death in many although not all circumstances. Thus YVAD-fmk completely blocks death of *Drosophila* cells induced by hyperexpression of *reaper* (*Pronk*, 1996). Ac-YVAD-CHO inhibits chicken motoneuron death induced by withdrawal of muscle-derived trophic factor (*Milligan,* 1995). It also blocks Fas-mediated apoptosis in many cell types (*Enari,* 1995; *Los,* 1995). However, apoptosis of macrophages induced *in vitro* by treatment with 5mM ATP is not inhibited by YVAD-cmk under conditions in which processing of proIL-1β is completely abrogated (*Nett-Fiordalisi,* 1995), suggesting that proteases of different specificity from ICE may be responsible for this apoptosis. In support of this, there is evidence for preferential inhibition of other proteases in the ICE family by other peptides. Thus Ac-DEVD-COOH contains the protease cleavage site in poly (ADP-ribose) polymerase (see below) and a biotinylated form of this tetrapeptide has been used as an affinity ligand to purify CPP32 from cytosolic extracts of the same cell type from which ICE was purified by YVAD affinity chromatography (*Nicholson,* 1995). These experiments thus suggest that whilst proteases identical or close to ICE in structure may be responsible for the effector process in Fas-induced killing, other triggers of death may involve other proteases. In theory different cell types may possess their own proteases, or there may be a cascade in which activation of one protease (eg. ICE) leads to the activation of other family members, and different stimuli trigger different elements of the cascade. Equally, it is possible that redundant parallel pathways exist of differing relative importance in different cell types. Unfortunately these issues are unlikely to be clarified by use of the inhibitors alone, because complete specificity of inhibition to any one family member is improbable given the known homologies of the substrate binding site.

Knock-out mice

Insight into the potential redundancy of proteases has been gained from observations in ICE knock-out mice. These develop normally, appear healthy and are fertile. Their macrophages are defective in processing and release of IL-1β but sensitive to ATP-induced apoptosis. Similarly thymocytes from ICE-knock-out mice have been shown to be sensitive to dexamethasone and radiation induced apoptosis (*Kuida,* 1995; *Li,* 1995). Hence none of these stimuli has an absolute requirement for ICE in engendering apoptosis in these cells. In contrast thymocytes from these ICE knock-out mice are resistant to anti-Fas triggered apoptosis, establishing a unique role for ICE in Fas-mediated apoptosis. Various models can be built around these results, eg. a linear 'cascade' in which ICE activated by Fas is 'upstream' of other proteases, or two convergent pathways, one involving ICE and triggered by Fas, the other accounting for other stimuli for death. These models could be definitively tested using knock-outs of various other proteases (Figure 2).

Cell free systems

Cell free systems provide another powerful approach for study of the effector mechanism of apoptosis. They allow dissection of the activation of the effector from the target structures involved in the effector pathway itself. Proteases or their inhibitors can be directly

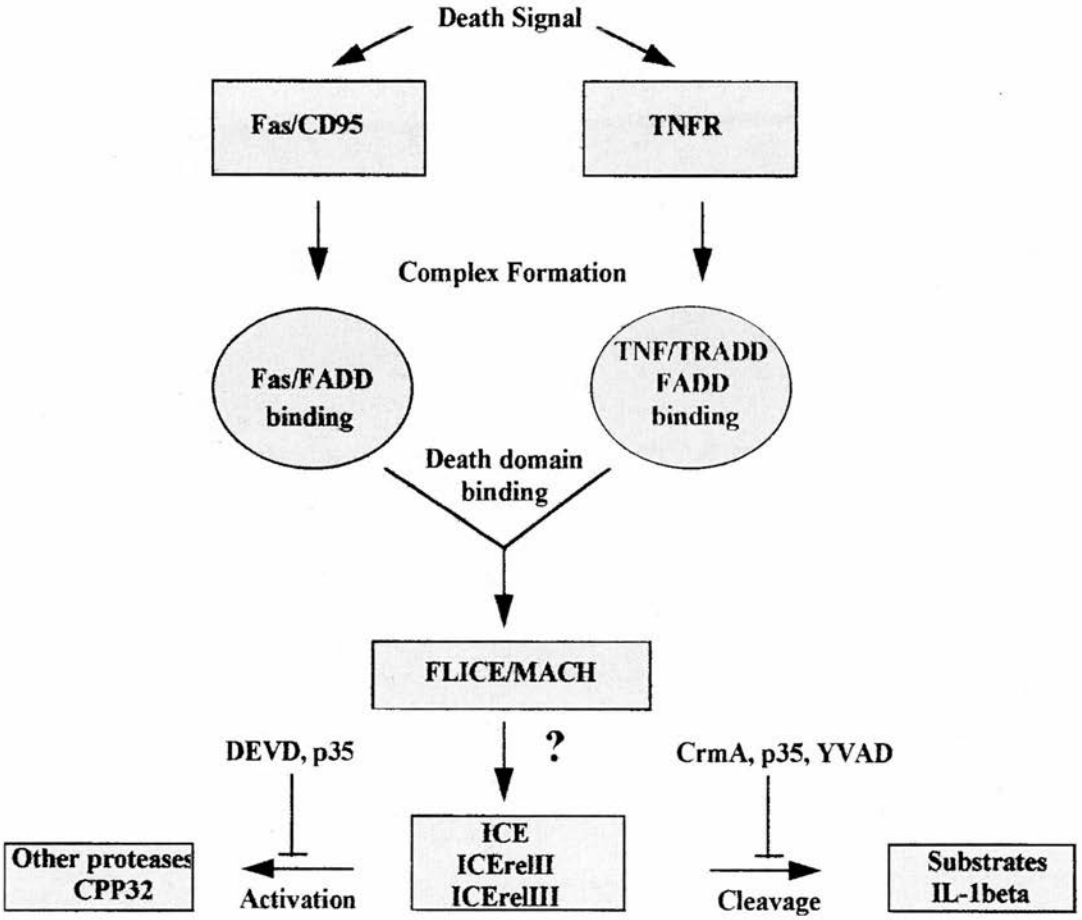

Fig. 2: Model for Fas and TNF induced apoptosis.

added to the system circumventing the permeability barrier that the plasma membrane presents in intact cells. Cell free systems thereby permit analysis of the role of individual components of the pathway and provide information about the identity and cellular location of the substrates of the effector. In the first description of such a system extracts from chicken hepatoma cells induced in HeLa nuclei all the nuclear morphological changes expected in apoptosis, including chromatin condensation, dissociation of the nuclear membrane and internucleosomal DNA fragmentation (*Lazebnik,* 1993). The active principle turned out to be a cytoplasmic cysteine protease named prICE (*p*rotease *r*esembling *ICE*). Its inhibition by TLCK (tosyl-L-lysyl chloromethylketone) and YVAD-cmk and its inability to cleave proIL-1β proved it could not be ICE itself (*Lazebnik,* 1994). Several other cell systems have since been described. They have not only established that activation of ICE family proteases is a key event during apoptosis but have also led to the discovery of downstream targets of these proteases (*Newmeyer,* 1994; *Lazebnik,* 1995; *Enari,* 1995; *Martin,* 1995, 1996).

Targets for ICE-Like Proteases

Study of the proteolytically cleaved proteins that appear during apoptosis has helped define

the biology of the effector pathway and has provided intriguing if still not definitive information on the plurality of the apoptotic proteases themselves. If the ICE-like proteases are indeed in the final common path for apoptotic effector events then their substrates should include cell components that are responsible for the structural changes observed in apoptotic cell death. Candidate substrates, known to undergo specific proteolytic cleavage in apoptosis, include PARP (*Kaufmann,* 1993; *Lazebnik,* 1994), nuclear lamins (*Lazebnik,* 1995), fodrin (*Martin,* 1995), U1 small nuclear ribonucleoprotein particle (U1 70) (*Casciola-Rosen,* 1994), growth arrest-specific protein2 (Gas2) (*Brancolini,* 1995), actin (*Mashima,* 1995; *Kayalar,* 1996), protein kinase Cδ (PKCδ) (*Emoto,* 1995), sterol regulatory element-binding protein (SREBP-1 and SREBP-2) (*Wang,* 1995, 1996), GDP dissociation inhibitor (D4-GDI) (*Na,* 1996), Retinoblastoma protein (RB protein) (*An,* 1996), the nuclear proteins DNA-dependent protein kinase (*Casciola-Rosen,* 1995), histone H1, topoisomerase I and II (*Kaufmann,* 1989; *Voelkel-Johnson,* 1995) and the cell membrane associated adenomatous polyposis coli protein (APC) (*Browne,* 1994).

Proteolytic cleavage of PARP

Proteolytic cleavage of PARP was originally discovered in etoposide-induced apoptosis and is an early event in apoptosis (*Kaufmann,* 1989; *Althaus,* 1987). PARP is a chromatin-associated protein with a molecular weight of 116 kDa that catalyses the covalent attachment of ADP-ribose units from its substrate NAD^+ to numerous nuclear proteins including itself. The enzyme is strongly stimulated by DNA strand breaks and activates DNA ligase II. Internucleosomal DNA degradation, characteristic of apoptosis, would thus be expected to activate the enzyme resulting in religation of the DNA fragments and consumption of NAD either of which could have serious sequelae in aborting the process of apoptosis. PARP is inactivated in apoptosis, however, by cleavage into two fragments of about 25 and 85 kDa, which isolates the DNA-binding from the catalytic domain of the protein (*Lazebnik,* 1994). The link between PARP cleavage and ICE-like proteases was established through the study of cell free systems.

Candidate proteases for PARP cleavage

PrICE was the first ICE-like protease shown to cleave PARP, producing a fragment of approximately 85 kDa. This activity is inhibited by decamer peptides that span the PARP cleavage site (*Lazebnik,* 1993). Several other ICE-like proteases have since been shown to have PARP cleavage activity *in vitro.*

CPP32, originally cloned from human Jurkat T-lymphocytes was shown to be responsible for PARP cleavage, both in a purified system with recombinant CPP32 and purified PARP and an overexpression system in stably transfected MCF7 breast carcinoma and BJAB lymphoma cell lines (*Fernandes-Alnemri,* 1994; *Tewari,* 1995). Both cleavage of PARP and apoptosis were inhibited by CrmA (*Tewari,* 1995). The role of CPP32 as the PARP cleaving enzyme seems to be further confirmed by several independent lines of evidence. The tetrapeptide aldehyde inhibitor Ac-DEVD-CHO blocks PARP cleavage in osteosarcoma cell extracts and attenuates the occurence of nuclear changes driven by THP-1 cell extract in a cell-free system (*Nicholson,* 1995). A cell-free system derived from anti-Fas treated Jurkat cells (Fas extract) led to the partial characterisation of an ICE-like protease which promotes apoptotic changes in isolated thymocyte nuclei (*Schlegel,* 1995). Further characterisation of these cytosolic extracts by immunoblotting with PARP specific antibody and rabbit polyclonal antibodies specific for the p17 and p12 subunits of CPP32 showed that CPP32 activation, from

its inactive proenzyme form to the active p17 and p12 subunit mature enzyme, correlated with the onset of PARP cleavage. In the presence of the tetrapeptide inhibitor Ac-DEVD-CHO this cleavage was completely inhibited, whereas CrmA did not interfere with either PARP cleavage activity or nuclear degradation activity (*Schlegel,* 1996).

There is also some evidence suggesting that other members of the ICE-like protease family may be involved in PARP cleavage. Coexpression of human PARP with human ICE, or the ICE homologs TX and NEDD-2 in COS cells resulted in PARP cleavage, inhibited by coexpression of CrmA (*Gu,* 1995). A comparison of protein concentration, however, showed that for ICE to cleave PARP a 50-100-fold higher protein concentration is required than to process proIL-1β. Coexpression of CMH-1, (standing for CPP32/*M*ch2 *h*omolog), in COS cells together with a truncated 45 kDa PARP polypeptide resulted in PARP cleavage to a 31 kDa peptide similar to that produced by ICE, TX and NEDD-2 (*Lippke,* 1996). CMH-1 was found to cleave PARP at less than 3nM concentration within 30 min. hence showing similar kinetics to PARP cleavage by CPP32 in the Fas cell-free system. No cleavage of proIL-1β was found, indicating that CMH-1, like CPP32, has a preferred substrate specificity for PARP (*Lippke,* 1996). Mch3 and ICE-LAP-3 (standing for *ICE-l*ike *a*poptotic *p*rotease 3), represent the same protease independently cloned by two groups and this also has been shown to inactivate PARP. Structurally Mch3 is the most closely related protease to CPP32 (58% sequence identity). Mch-3(ICE-LAP-3), cloned from human Jurkat T cells has been shown to cleave PARP in less than 15 min. when incubated with either purified bovine PARP or human Hela nuclei and this activity is only weakly CrmA inhibitable (*Fernandes-Alnemri,* 1995).

Two other ICE-like proteases have PARP cleavage specificity. CED-3 cleaves PARP into products indistinguishable from the products generated by CPP32 and this cleavage is inhibited by baculovirus p35 protein (*Xue,* 1995; *Hugunin,* 1996). Mch2 *m*ammalian, CED-3 *h*omolog) with 38% sequence identity with CPP32 and 35% identity with CED-3 cleaves PARP to generate a major product of approximately 83 kDa and a minor band at about 57 kDa. In contrast PARP cleavage by CPP32 at the DEVD site results in fragment of 89.3 kDa predicted molecular mass. This suggests that Mch2 cleaves PARP at a site COOH-terminal to the DEVD site (*Duan,* 1996). This is consistent with the observation that recombinant CPP32 cleaves synthetic DEVD with a higher activity than Mch2.

Hence, CPP32 and the closely related ICE family members (Mch2, Mch3 and CMH1) are good candidates for the enzymes that cleave PARP early in apoptosis. The question arises whether PARP cleavage by CPP32 is *essential* for apoptosis. This appears unlikely since PARP knock-out mice are healthy and fertile, with only a susceptibility to develop skin disease, and without major disruption in their patterns of developmental cell death (*Wang,* 1995). Moreover, CPP32 in isolation cannot provoke apoptotic changes when added to healthy nuclei, showing that it is not sufficient for induction of apoptosis (*Nicholson,* 1995). CPP32 is thus likely to act in conjunction with other family members in the apoptotic pathway and has been shown to have at least one other substrate.

Multiple ICE-like proteases with capacity for cleavage and inactivation of PARP not only exist but can exist together in a single cell. This suggests some functional redundancy between individual family members but also points towards the existence of other substrates which are not necessarily directly involved in cell death. This redundancy might be expected as it provides comprehensive protection of cells against viral inhibitors and so protects tissues against clonal expansion or lysis of virus infected cells.

Further substrates

Amongst other substrates cleaved by ICE-like proteases are the nuclear lamins. Lamins are intermediate filament proteins localized at the nuclear envelope and probably involved in nuclear envelope integrity and in organisation of chromatin in interphase (*Lazebnik,* 1993). Proteolytic cleavage of lamins A, B and C can be reproduced by exposing normal nuclei to cell free extracts with ICE-like protease activity. Lamin proteolysis begins about ten minutes after proteolysis of PARP has been completed and has a different inhibitor profile, being sensitive to inhibition by TLCK (tosyl-L-lysyl chloromethylketone) and up to 100-fold more sensitive to YVAD-cmk than PARP (*Lazebnik,* 1995). Interestingly, addition of TLCK to S/M extracts inhibits the formation of apoptotic bodies but allows chromatin condensation and DNA fragmentation to occur. Hence, it is probable that lamin and PARP cleavage are mediated by different enzymes, with preferential control over different elements of the process of apoptosis and the PARP cleaving enzyme acting earlier than the lamin-cleavage enzyme. Very recent data seem to confirm this as Mch2 (but not CPP32) has been shown to cleave lamin A (*Takahashi,* 1996).

Fodrin is a cytoskeletal component cleaved during apoptosis induced by a variety of stimuli such as ligation of the CD3/T cell receptor complex, Fas activation and treatment of cells with staurosporine, glucocorticoid or synthetic ceramide. Fodrin or nonerythroid spectrin is a multifunctional protein and a major component of the cortical cytoskeleton. The protein is composed of two subunits (alpha and beta). The alpha subunit (molecular weight approximately 240 kDa) is cleaved in apoptotic cell extracts to a major detectable band of about 150 kDa (*Martin,* 1995). Cytoplasmic extracts of UV-irradiated or anti-Fas-treated cells similarily contain a fodrin-cleavage protease (*Martin,* 1995). It is not yet established whether this cleavage is the direct consequence of the activation of an ICE-like protease or whether it is secondary to proteolytic activation of the calcium-dependent neutral protease calpain, whose *in vitro* substrates include cytoskeletal proteins. Membrane blebbing in apoptosis could be the consequence of the disruption of the fodrin network.

Another component of the microfilament system, Gas2, has been shown to be cleaved in nonadherent COS7 cells induced to undergo apoptosis by serum withdrawal (*Brancolini,* 1995). Gas2 is a 35 kDa protein whose tissue specific expression is increased during growth arrest. It can induce alterations of the actin cytoskeleton and of cell shape and this ability is associated with proteolytic cleavage at an aspartic acid residue near the C-terminus to a 31 kDa protein. This implies not only that Gas2 is a potential target for an ICE-like protease but also provides a mechanistic connection between apoptotic proteases and cell shape.

Actin may also be a target for ICE-protease activity, as it is cleaved from its 42 kDa native form to 15 and 30 kDa fragments in human myeloid leukemia U937 cells induced to enter apoptosis by the chemotherapeutic agent VP-16 (*Mashima,* 1995). Purified G-actin incubated with purified ICE results in cleavage into 41, 30 and 14 kDa fragments. Actin cleavage in apoptosis results in prevention of polymerisation to its filamentous form and in incapacity to inhibit DNaseI. In this way actin cleavage could be linked directly to plasma membrane blebbing and oligosomal DNA fragmentation (*Kayalar,* 1996).

Another potential substrate is the U1 small nuclear ribonucleoprotein particle which is essential for splicing of precursor mRNA and is cleaved from a 70 KDa fragment to a 40 KDa fragment in Hela cells induced to undergo apoptosis by serum withdrawal and UV irradiation (*Casciola-Rosen,* 1994). This cleavage and the apoptotic morphology are inhib-

ited by iodoacetamide, TPCK and TLCK, an inhibitor profile identical to that of ICE. Studies on Fas- and TNF-induced cell death in human lymphoma and breast carcinoma cell lines reveal that cleavage of U1-70 kDa is inhibited by CrmA and correlates in time course with PARP cleavage (*Tewari,* 1995). A possible role of U1-70 cleavage in apoptosis might be the regulation of RNA splicing.

Recent studies on protein kinaseCδ (PKCδ), a cytoplasmic myelin basic protein kinase, demonstrate that the 78 kDa intact enzyme is cleaved to a 40 kDa C-terminal fragment after ionizing radiation of human U-937 cells (*Emoto,* 1995). This cleavage which results in activation of the enzyme occurs adjacent to an aspartic acid residue and is inhibited by preincubation of the cells with the ICE protease inhibitor YVAD-cmk. Recombinant ICE, however, is unable to cleave the enzyme which suggests the involvement of an ICE-like protease other than ICE in the proteolytic activation of PKCδ. The sterol regulatory element binding-proteins are another substrate cleaved both *in vitro* and *in vivo* by CPP32. Cleavage is inhibited by the tetrapeptide Ac-DEAD-CHO. The physiological function of such cleavage, however, is unknown (*Wang,* 1995, 1996).

Cleavage of cytoskeletal protein, such as fodrin and actin, and GAS2 activation could therefore all contribute to the morphological changes and rearrangements characteristic of apoptosis. Several other proteins appear to undergo site specific proteolytic cleavage in apoptosis even though it is not yet clear whether an ICE family member is directly involved. These include the nuclear proteins histone H1, topoisomerase I and II (*Kaufmann,* 1989; *Voelkel-Johnson,* 1995), D4-GDI (*Na,* 1996), RB protein (*An,* 1996) and adenomatous polyposis coli protein (*Browne,* 1994). There is also exciting evidence that TNF and fas stimulation can activate acid sphingomyelinase (*Wiegmann,* 1994; *Grazia Cifone,* 1994) so demonstrating a connection between these signalling pathways and ceramide production. Evidence that sphingomyelinase activation is mediated by proteolytic cleavage is still to be published.

It is of course possible that some of these proteolytic events represent a terminal state of chaos in the dying cell, in which enzymes are able to access and cleave proteins that are not their natural substrates. The roles played by apoptosis in biology and all that is currently known for certain of the apoptosis proteases, however, suggests another story: that apoptosis is an entirely orderly process in which critical elements in the cell are dismantled in a predetermined sequence. While some events appear to be triggered in parallel (eg. U1-70 and PARP cleavage) others may be in series (eg. PARP and lamins), possibly because of a cascade-like central organisation of the ICE family protease activation. Recent studies on ICE and CPP32 activation in Fas-induced apoptosis seem to be in favour of a cascade-like organisation of the ICE-like proteases. Kinetic studies demonstrate an ICE-like protease activity before CPP32-like activity and peptide inhibitor studies indicate that the latter activity depends on previous ICE-like activity (*Enari,* 1996). The ICE-like proteases thus have the capacity to contribute to multiple near-synchronous events in the nucleus and the cytoplasm that could underlie the long-known characteristic structural events of apoptosis. How this cleavage activity of the ICE-like proteases is regulated is still one of the poorly understood phenomena of apoptosis (Figure 3).

Regulation of the ICE-Like Proteases

Application of both cell-permeable synthetic peptide and natural, macromolecular inhibitors as well as the identification of several potential downstream targets have established ICE-like proteases as effector molecules of the apoptotic pathway. How this protease activity is activated and regulated and where

the ICE-like enzymes act in the pathway in relation to other important regulatory genes are only partially resolved. Multiple levels of control probably exist for the regulation of ICE protease activity within the cell. Control could be achieved at the mRNA level through transcriptional regulation and through alternative splicing, at the protein level through post-translational modification and proteolytic cleavage, through interaction with other components of the cell death pathway which either activate or inhibit the ICE-like proteases and by autocatalysis and interfamily interactions.

Northern blots demonstrate that mRNA expression of all ICE-like proteases can be detected in most human tissues. ICE mRNA has been shown to be induced by loss of adhesion to extracellular matrix and by transcription factor IRF-1 (*Boudreau,* 1995; *Tamura,* 1995). Northern blotting analysis further reveals the presence of splice variants of some of the family members. Four isoforms of ICE have been identified and cloned, named ICE β, χ, δ and ϵ (*Alnemri,* 1995). Isoforms of Mch2 (Mch2α and β), ICH-1/NEDD-2 (termed ICH-I_L and ICH-1_S) and Mch3 splice variants α and β have also been described (*Fernandes-Alnemri,* 1995; *Wang,* 1994). These isoforms arise by the usage of alternative splice donor/acceptor sites and result in an α/L full length isoform of the enzyme and a truncated β/S form of the enzyme. Expression of the ICH-1_S in Rat-1 cells was shown to prevent cell death induced by serum withdrawal (*Wang,* 1994). Potentially these isoforms could regulate the enzyme activity by acting as dominant negative inhibitors and interfere with the heterodimerization of the active full length form of the enzyme thereby forming inactive enzyme. The role of the ICH-1_S isoform of the enzyme as an inhibitor of cell death is, however, controversial as transfection of FDC-p1 cells with NEDD-2s, the mouse equivalent of ICH-1_S, had no effect on cell survival and therefore did not suggest a major role in the regulation of apoptosis at least in that cell type (*Kumar,* 1995).

So far no information is available on post-translational glycosylation or phosphorylation of the ICE-like proteases. Post-translational activation by proteolytic cleavage, however, is a general feature of the enzyme family as decribed earlier. Most members of

	ICE-like proteases								
Substrates	CED3	ICE	CPP32	ICH-1/NEDD2	Mch2	Mch3	CMH-1	TX/Ich-2/ICErelII	ICE-like protease
pro IL-1beta	*	+							
PARP	+	*	+	*	+	+	+	+	
actin		*							+
SREBPS			+						
GAS2									+
APC									+
RB protein									+
D4-GDI									+
PKC									+
fodrin									+
U1snRNP									+
other nuclear (DNA dep.PK, histone H1, tipoisomerase I + II)									

\+ = cleavage
* = cleavage at high substrate concentration

Fig. 3: Substrate cleavage by ICE-like proteases.

the family exhibit autocatalytic cleavage and activation when overexpressed in bacteria as proenzymes. The extent to which this autoactivation in authentic apoptosis occurs is still uncertain, and activation of one family member by another in a cascade, as discussed above, is also an attractive possibility. Thus TX when overexpressed in COS cells has protease activity against the ICE precursor (*Faucheu,* 1995). Mch3α has a potential cleavage site (amino acids 20-23, DSVD) which is very similar to the CPP32 tetrapeptide substrate DEVD, making Mch3α a possible substrate for CPP32 (*Fernandes-Alnemri,* 1995). CPP32 itself does not appear to undergo autocatalytic cleavage. Its activation therefore requires another aspartic acid specific protease. Purified ICE and FLICE have both been shown to cleave CPP32 *in vitro,* indicating that they may lie upstream of CPP32 in the cascade (*Tewari,* 1995). The serine protease granzyme B from the granules of cytotoxic T lymphocytes (CTL) also activates CPP32 and cleaves ICE (*Darmon,* 1995; *Quan,* 1996). Cleavage of ICE by granzyme B, however, does not lead to ICE activation (*Darmon,* 1994, 1995; *Quan,* 1996; *Martin,* 1996). This not only suggests a role for CPP32 and perhaps other ICE proteases in CTL mediated cytotoxicity but also implies that various cell death pathways, including granzyme B induced apoptosis, converge into the same effector pathway. These findings, together with the data discussed earlier that indicate differing substrate specificities for the ICE family, support the concept that apoptosis is effected by a central proteolytic cascade whose action in sequence on substrates throughout the cell effect its orderly dissassembly. Granzyme B gives the first indication that other non ICE-like proteases interact and are part of this cascade. Studies on thymocyte apoptosis induced by diverse stimuli confirm that proteases of differing specificities are required for the complete execution of apoptosis as different inhibitors with a variety of specificties inhibit the apoptotic process at various points (*Fearnhead,* 1995; *Zhivotovsky,* 1995).

Further control of enzyme activity might be achieved through hetero-oligomerisation. Several members of the ICE-like protease family can heterodimerize with one another, such as CPP32 and Mch3 or ICE and TX, forming active heterodimeric enzyme complexes and so compensating for mutational inactivation of individual subunits (*Fernandes-Alnemri,* 1995; *Faucheu,* 1995; *Gu,* 1995). Oligomerisation between different ICE-like proteins might not only alter their substrate specificities but could potentially modulate their enzymic activity and stability.

Insight into the upstream regulation of the ICE-like proteases activity is still limited. Upstream regulation is likely to be achieved by members of the Bc1-2 family, the mammalian homologue of the nematode ced-9 negative regulator of cell death. Bc1-2 has been shown to effectively block cell death caused by overexpression of *ICH-1*$_L$ cDNA (*Miura,* 1993) and a variety of other apoptotic stimuli. Recent biochemical studies on Bcl-2, Bcl-xL and the adenovirus E1B 19 kDa protein, a viral homologue of Bcl-2, have, however, clarified the order of the cell death pathway components. Bcl-2 and Bcl-x_L have been shown to function upstream of the ICE-like proteases CPP32 and ICE-LAP3 in the pathway (*Chinnaiyan,* 1996) as overexpression of *bcl-2* or *bcl-x*$_L$ abrogates the proteolytic activation of these enzymes in staurosporine induced apoptosis. Similarly bcl-2 and adenovirus E1B kDa protein blockade the CPP32 activation, cleavage of PARP and apoptosis induced by E1A (*Boulakia,* 1996). This negative regulatory role of Bcl-2 and Bcl-x_L, however, seems to be dependent on the particular apoptotic stimulus as they are unable to inhibit Fas/APO-1 or TNF induced cell death. These pathways are inhibited by CrmA, preventing subsequent PARP cleavage and enzyme activation (*Duan,* 1996). Nevertheless, immunoblotting with antibodies to

active CPP32 and ICE-LAP3 has demonstrated that these ICE-like proteases are part of the response to staurosporine, Fas/APO-1 and TNF induced cell death. It thus appears that although different apoptotic stimuli activate the same effector molecules it is not necessarily by the same route, suggesting distinct apoptotic pathways in these systems. In the non Fas/APO-1/TNF cell death pathway bcl-2, bcl-x_L and E1B act either upstream or at the activation step of CPP32 and ICE-LAP-3 and likely other members of the family.

The most dramatic evidence for upstream regulation of the ICE-like proteases comes from very recent work on the proteins interacting with the cytosolic moiety of CD95/Fas/APO-1 and the TNFR. A new member of the ICE-like protease family, called FLICE/MACH contains a prodomain homologous to FADD and an ICE-like protease homology region corresponding to the active enzyme subunits. FLICE/MACH has been shown to interact with the receptor death domain of FADD/MORT1 and TRADD which in turn form a complex with activated Fas/Apo-1/CD95 or TNFR. Hence this recent work demonstrates a direct non-transcriptional pathway where Fas activation leads to triggering of the protease cascade (*Muzio*, 1996; *Boldin*, 1996)

Conclusion

In the nematode a mutation or deletion of a single gene, *ced-3*, abolishes cell death (*Ellis*, 1991). Regulation of cell death in higher organisms is substantially more complex. Not only are there multiple enzymes homologous to Ced-3, forming the family of ICE-like proteases, but these proteases appear to have overlapping substrate specificities and might all be involved in the apoptotic pathway in a single cell type. Confronted with such complexity much remains to be defined. Knockout mice, specific high titer antibodies and more selective inhibitors, as more substrates for other ICE family members are determined, are required to define the role of individual members. Despite its complexity and redundancy, the ICE-family protease pathway offers intriguing possibilities for specific, selective pharmacological interventions in the process of apoptosis.

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